

PHYSIOLOGICAL AND BIOCHEMICAL STUDIES OF
THE MECHANISM OF PARAQUAT RESISTANCE
IN AMERICAN BLACK NIGHTSHADE

By

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I dedicate this work to my nieces and nephews Siobhan, Suraya, Stephen and Matthew and to my brother Jonathan.

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PHYSIOLOGICAL AND BIOCHEMICAL STUDIES OF
THE MECHANISM OF PARAQUAT RESISTANCE
IN AMERICAN BLACK NIGHTSHADE

By

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Major Department: Horticultural Science

The primary mechanism of paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) resistance was investigated in American black nightshade (*Solanum americanum* Mill.). Physiological and biochemical studies were conducted with a paraquat-resistant (R) biotype and a paraquat-sensitive (S) biotype.

There was no difference in the amount of paraquat that penetrated to the cytoplasm. The R biotype exhibited greater apoplastic adsorption of paraquat than the S biotype; however, apoplastic sequestration is unlikely as a primary mechanism because there was 3-fold more paraquat in the cytoplasm than was adsorbed in the apoplast.

Paraquat treatment increased the rates of ethane production above control levels in both biotypes, but to a greater extent in S biotypes. An enzyme inhibitor

ensured that this was not due to elevated levels of radical scavenging enzymes. A larger difference between R and S biotypes in the rate of electrolyte leakage occurred in light than in darkness. Concentration-dependent studies of paraquat-induced ion leakage showed that the S biotype had higher ratios of $I_{50(\text{dark})}:I_{50(\text{light})}$ (I_{50} = paraquat concentration that caused 50% ion leakage) than the R biotype. Diquat (6,7-dihydrodipyrido[1,2- α :2',1'-c]pyrazinediium ion) and paraquat suppressed variable fluorescence (F_v) at the same rate, indicating rapid penetration to the site of action in both biotypes. However, the magnitude of F_v reduction was smaller in the R biotype, so that higher concentrations of diquat and paraquat were required to reduce F_v to a level similar to that of the S biotype. The electrolyte leakage and fluorescence studies support a light-dependent mechanism of resistance.

Electron transport rates, when measured as the photoreduction of ferricyanide and NADP^+ by isolated thylakoid membranes, were significantly higher in the S biotype. The potential of the two biotypes to generate oxidative stress was also compared. Nontreated thylakoids of the S biotype produced superoxide at a faster rate than those of the R biotype. The increase in the rate of superoxide formation resulting from paraquat treatment was larger in the thylakoids of S biotype than the R biotype. The data imply that paraquat resistance in American black nightshade is due to reduced photosynthetic electron flow.

CHAPTER 1 GENERAL INTRODUCTION

Chloroplast Susceptibility to Oxygen Toxicity

Illuminated chloroplasts are subject to oxidative stress for a number of reasons (Halliwell, 1987). The splitting of water to provide electrons to photosystem II (PSII) creates a high ambient oxygen (O_2) concentration in the chloroplast. Therefore, O_2 is readily available to participate in reactions that generate toxic derivatives. Excitation energy in illuminated chloroplasts that is not dissipated by electron transfer, fluorescence or heat can cause the formation of triplet chlorophyll. This type of chlorophyll can result in the formation of singlet oxygen, which can be extremely damaging to chloroplast membranes (Halliwell, 1987). Also, inefficiencies in photosynthetic electron transport lead to electrons being leaked to oxygen resulting in its univalent reduction to superoxide (O_2^-) (Asada et al., 1974). The reaction is apparently associated with the autooxidation of electron acceptors on the reducing side of photosystem I (PSI) and is considered to be a partial reaction of the Mehler reaction (Asada et al., 1974). A small amount of O_2^- is also formed when oxygen is reduced by ferredoxin (Allen, 1977) (Fig. 1). Chloroplast membrane lipids contain a large amount of polyunsaturated fatty acids that predispose them to lipid peroxidation. Therefore, the chloroplast is an organelle with inherent oxygen toxicity problems.

Protection against oxidative stress is provided by a battery of enzymes and antioxidants that can alleviate normal oxidative stresses.

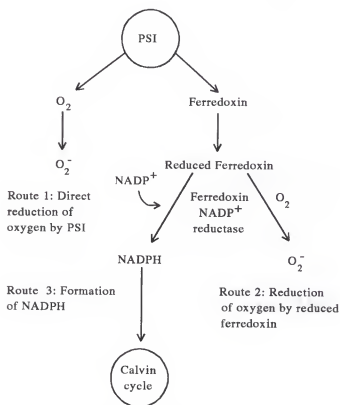


Figure 1. Alternative routes for electron flow beyond PSI. (Adapted from Halliwell and Gutteridge, 1989).

Halliwell and Gutteridge (1989) defined free radicals as chemical species capable of independent existence that possess one or more unpaired electrons. Such a condition usually results in a species that is more reactive than the parent atom or molecule. Radicals can potentially initiate a chain of reactions because the reaction of a radical with a nonradical always results in the production of another radical (Rabinowitch and Fridovich, 1983).

The superoxide radical can participate in both oxidation and reduction reactions (Rabinowitch and Fridovich, 1983). Superoxide may also initiate and propagate free radical chain reactions (Fridovich, 1988). Although O_2^- is not a very reactive radical, Fridovich (1986, 1988) regards it as capable of direct damage to cell constituents, and considers its innate toxicity to be underestimated while emphasis is placed on its indirect effects through the formation of hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\bullet$).

The chloroplast is protected from O_2^- in a number of ways. The radicals undergo spontaneous dismutation on collision with each other, yielding H_2O_2 . This reaction occurs at a much faster rate when catalyzed by superoxide dismutase (EC 1.15.1.1) (McCord and Fridovich, 1969).



Asada et al. (1973) demonstrated the presence of superoxide dismutase (SOD) in spinach chloroplasts. Although most of the activity is due to the stromal Cu, Zn-SOD, spinach chloroplasts also contain a membrane-bound SOD. Superoxide is also scavenged by ascorbate (Nishikimi and Yagi, 1977).

Although H_2O_2 is not a radical, it is a substantial contributor to oxygen toxicity in the chloroplast. A strong oxidant, H_2O_2 oxidizes several of the Calvin cycle enzymes and inhibits carbon dioxide fixation (Kaiser, 1979; Law et al., 1983). It inhibits SOD and can be reduced in an iron-catalyzed Haber-Weiss reaction to produce the $OH^\bullet$ (Halliwell and Gutteridge, 1989).

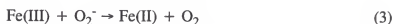


The scavenging of H_2O_2 in the chloroplast was found to be catalyzed by a peroxidase with ascorbate as the electron donor (Nakano and Asada, 1980, 1981). Subsequently, it was shown that the addition of H_2O_2 to intact chloroplasts resulted in a decrease in ascorbate content (Foyer et al, 1983; Law et al. (1983). Ascorbate peroxidase (EC 1.11.1.7) is the first of a series of enzymes that comprise the ascorbate-glutathione cycle or Halliwell-Asada pathway (Fig. 2). Dehydroascorbate reductase (EC 1.8.5.1) is the next in the series and catalyzes the regeneration of ascorbate from dehydroascorbate with reducing power provided by reduced glutathione (GSH) (Nakano and Asada, 1981). This reaction also occurs spontaneously at physiological pH (Foyer and Halliwell, 1976). Oxidized glutathione (GSSG) is reduced to GSH in the presence of glutathione reductase (EC 1.6.4.2) and NADPH (Foyer and Halliwell, 1976). Ascorbate and GSH have been detected in the chloroplast at millimolar concentrations (Foyer et al., 1983; Law et al., 1983).



Figure 2. Ascorbate-glutathione cycle (adapted from Shaaltiel et al., 1988b). The enzymes designated 1, 2, 3 and 4 are SOD, ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase, respectively.

As discussed previously, O_2^- and H_2O_2 can react in the presence of iron complexes to give $OH^\bullet$ (Rabinowitch and Fridovich, 1983). Equations 3 and 4 are the partial reactions that result in the net reaction shown in Equation 2.



Equation 4 is known as the Fenton reaction. The source of the iron *in vivo* may be phytoferritin (Dodge, 1989). Saito et al. (1985) have shown that O_2^- can cause the release of iron from ferritin. The production of $OH^\bullet$ has been demonstrated in isolated thylakoids by detecting the formation of ethylene from methional (Elstner and Konze, 1974).

Hydroxyl radicals are extremely reactive. They react with almost all cell constituents: phospholipids, nucleotides, amino acids, sugars and organic acids (Halliwell and Gutteridge, 1989). The reactions are mainly hydrogen (H) abstraction, addition, and electron transfer. Hydrogen abstraction is particularly important since it is the means by which the peroxidation of cell membrane lipids is initiated (Halliwell and Gutteridge, 1989). Hydroxyl radicals are scavenged by ascorbate, GSH and α -tocopherol (Dodge, 1989).

Lipid Peroxidation

Lipid peroxidation is the process in which unsaturated lipid molecules undergo oxidative decomposition by means of a series of free-radical chain reactions. Two

types are recognized: enzymic and nonenzymic. Only the nonenzymic process is pertinent and will be described briefly. Gutteridge (1988) and Halliwell and Gutteridge (1989) may be consulted for a more comprehensive discussion. Chain initiation occurs when a free radical with sufficient reactivity abstracts a H atom from a methylene carbon of an unsaturated fatty acid. This results in a carbon radical that undergoes stabilization to form a conjugated diene. Conjugated dienes react with molecular oxygen giving rise to peroxy radicals. Peroxy radicals are important in the propagation of the free-radical chain reaction because they stimulate the peroxidation process by abstracting H atoms from adjacent fatty acid chains. Lipid hydroperoxides are formed when the peroxy radical combines with the abstracted H atom. Termination reactions are also characteristic and these involve the reaction of two radicals to form a nonradical (Gutteridge, 1988; Halliwell and Gutteridge, 1989).

Although $\text{OH}^\bullet$ radicals can initiate lipid peroxidation, $\text{O}_2^\cdot$ radicals are not sufficiently reactive and their charge precludes them from easily entering hydrophobic lipid bilayers. The protonated form $\text{HO}_2^\bullet$ (the hydroperoxyl radical) is much more reactive and, being neutral, easily penetrates membranes. It has been shown to initiate peroxidation of fatty acids *in vitro*. However, at physiological pH the charged species predominates and no evidence has yet been provided that either species directly induces lipid peroxidation *in vivo* (Halliwell and Gutteridge, 1989).

Bipyridinium Herbicides

Chemical Structure and Herbicidal Activity

The herbicidal properties of the bipyridiniums (bipyridyliums) were discovered in 1955 (Brian et al., 1958). Currently only diquat (6,7-dihydrodipyrido[1,2- α :2',1'-c]pyrazinediium ion) and paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) are in commercial use as herbicides (Fig. 3). They are commonly synthesized as dibromide and dichloride salts, respectively. However, only the cation is phytotoxic (Homer et al., 1960).

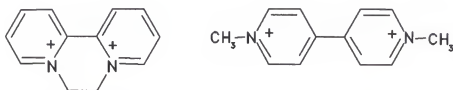


Figure 3. 6,7-dihydrodipyrido[1,2- α :2',1'-c]pyrazinediium ion (diquat) and 1,1'-dimethyl-4,4'-bipyridinium ion (paraquat).

Summers (1980) reviewed the chemistry and other significant features of the bipyridiniums. Paraquat is a diquatery salt of 4,4'-bipyridine. It is comprised of two pyridine rings, aromatic rings in which one carbon has been replaced by nitrogen (N), linked at carbon 4. The term quaternary refers to the characteristic bonding of the N atoms with the alkyl groups that results in a divalent cation (Halliwell and Gutteridge, 1989). Diquat is a bridged diquatery salt of 2,2'-bipyridine. The pyridine rings are joined at carbon 2, and at the two N atoms with an ethylene bridge.

Paraquat salts were first synthesized in the late nineteenth century (Summers, 1980) and, as methyl viologen, have been used as oxidation-reduction indicators since 1932 (Michaelis and Hill, 1933). In the 1950s, it was determined that diquat was a fast-acting, nonselective herbicide with desiccating action (Brian et al., 1958). Primarily a contact herbicide, limited translocation was observed in the shoot. Uptake was rapid, so that rain just after application did not reduce control. The herbicide is inactivated in soil due to adsorption to soil colloids (Brian et al., 1958).

The bipyridiniums vary in herbicidal activity. The requirements for herbicidal activity have been determined empirically. As compounds with low redox potentials, they can undergo a single electron reduction (Michaelis and Hill, 1933).



Only those compounds that can undergo a reversible univalent reduction to a stable radical possess herbicidal activity (Homer et al., 1960). Radical stability is characteristic of bipyridyls in which the pyridine rings are held in a coplanar configuration. Stability is due to the ability of the radical to exist in a number of resonance forms (Homer et al., 1960). The degree of activity has been shown to be related to the redox potential of the compound. Compounds with lower potentials required higher concentrations than those of higher potential to produce an equivalent effect (Black, 1966; Homer et al., 1960; Kok et al., 1965). The highest herbicidal activity occurs in bipyridyls with redox potentials in the range -0.3 to -0.5 V (Summers, 1979). The redox potentials of paraquat and diquat are -0.35 and -0.45 V,

respectively (Homer et al., 1960). The size of the ion is also important because, only compact molecules possess herbicidal activity (Summers, 1979).

Mechanism of Action

Mees (1960) showed that diquat had very slow herbicidal activity in darkness but phytotoxicity in light was accelerated. He hypothesized that this pointed to the involvement of the photosynthetic apparatus. Support for this hypothesis was provided by Davenport (1963) who showed that several bipyridyls catalyzed photophosphorylation in isolated chloroplasts. Zweig et al. (1965) observed that diquat inhibited the reduction of NADP^+ and proposed a site of action at the reducing end of PSI. The site has been located more precisely at the terminal electron acceptors F_A/F_B (Parrett et al., 1989). Fujii et al. (1990) have shown that F_B is the main site of electron donation to paraquat and that F_A has a lower affinity for the herbicide (Fig. 4).

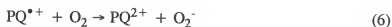


Figure 4. Scheme depicting the electron acceptors of PSI (Adapted from Dodge, 1989).

Harris and Dodge (1972a) found that continuous illumination of paraquat-treated flax (*Linum usitatissimum* L.) cotyledons resulted in the rupture of the tonoplast. This was followed by damage to mitochondrial and chloroplast

membranes. Membrane damage was also observed in dark-incubated tissue after 30 h. Further investigation provided more evidence of membrane injury in the presence of paraquat (Harris and Dodge, 1972b). There was an increased rate of ion leakage from tissue slices, enhanced accumulation of malondialdehyde and a progressive change in lipid composition.

Baldwin (1969) concluded, NADPH deprivation due to diversion of electron transport by paraquat, would result in a slow phytotoxicity similar to herbicides that inhibit electron transport in PSII. Such deprivation does not explain the rapid wilting, chlorosis and death of plants that commonly result from application of a bipyridyl herbicide. Calderbank (1968) predicted the involvement of oxygen radicals in the mode of action of bipyridyls. Farrington et al. (1973) felt that the membrane damage observed by Harris and Dodge could be due to paraquat-derived toxic oxygen radicals that were sufficiently long-lived to reach and react with membrane constituents. It has now been established that the bipyridyl radicals react with O_2 to produce O_2^- (Asada and Kiso, 1973; Stancliffe and Pirie, 1971).



Using pulse radiolysis, Farrington et al. (1973) determined the rate constant of the reaction of the paraquat radical with O_2 to be $7.7 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and proposed that O_2^- was a good candidate for mediating paraquat toxicity. Evidence that O_2^- is generated in paraquat-treated leaves was provided when a D-penicillamine-copper chelate was shown to inhibit paraquat toxicity in flax cotyledons (Youngman et al., 1979). This

compound is capable of the dismutation of O_2^- (Youngman et al., 1979). It is estimated that paraquat increases O_2^- production 10- to 20-fold, which is thought to be sufficient to overwhelm the plant's protective mechanisms (Asada et al., 1977).

Carbon fixation in flax cotyledons was totally inhibited by paraquat by 5 h after treatment (Harris and Dodge, 1972b). This is consistent with the diversion of electrons from NADPH formation (Zweig et al., 1965) and the inactivation of Calvin cycle enzymes by H_2O_2 (Kaiser, 1979). H_2O_2 is thought to be produced directly during the autooxidation of bipyridinium radicals under aerobic conditions, as well as indirectly via the spontaneous and catalytic dismutation of O_2^- (Black, 1966; Davenport, 1963; Kok et al., 1965). The addition of paraquat to illuminated chloroplasts resulted in oxidation of GSH to GSSG and a net loss of ascorbate (Foyer et al., 1983; Law et al., 1983). This provided evidence that paraquat action produces toxic oxygen species that require scavenging by these antioxidant molecules, and also that the major function of the enzymes of the ascorbate-glutathione cycle was to eliminate H_2O_2 in the chloroplast.

Paraquat-treated chloroplasts have also been shown to generate the $OH^\bullet$. Initially, only the electron spin resonance signal of O_2^- was detected. On addition of SOD, the signal changed to that of $OH^\bullet$ (Dodge, 1989). Babbs et al. (1989) have detected $OH^\bullet$ in paraquat-treated plants at levels that were considered to be lethal. A reaction proposed by Winterbourn (1981) between the paraquat radical and H_2O_2 giving rise to the hydroxyl radical in the absence of a metal catalyst has since been discounted (Halliwell and Gutteridge, 1989). Other highly reactive species have been

proposed to be generated in the presence of paraquat. These include 'crypto-OH' (Youngman and Elstner, 1981; Youngman, 1984) and an iron (IV) species (Sutton et al., 1987).

In summary, paraquat applied to illuminated plants is reduced by PSI, which decreases the NADPH concentration and forms paraquat radicals. Under the aerobic conditions of the chloroplast, the paraquat radicals are instantly reoxidized by O_2 which generates O_2^- . Superoxide is dismutated either spontaneously or by SOD to form H_2O_2 . The H_2O_2 not only blocks carbon fixation but also affects the ascorbate-glutathione pathway since there is little reducing power for the functioning of glutathione reductase. The enhanced production of O_2^- and H_2O_2 and their impaired scavenging results in the formation of $OH^\bullet$. The indiscriminate reactivity of $OH^\bullet$ radicals leads to the destruction of cell constituents. One major consequence is the peroxidation of cell membranes, including the tonoplast. There is a loss of osmotic control and fluctuation in pH. The rupture of the tonoplast causes the release of the toxic contents of the vacuole contributing to the general deterioration of the cell (Dodge, 1989).

Herbicide Resistance

With the increased occurrence of weed resistance to herbicides, reports on the subject in the literature have become more frequent. There has been a problem of inconsistency in the use of the terms tolerant and resistant. It was recently suggested within a committee of the Weed Science Society of America that the term resistance

should supersede tolerance. The decision on terminology has been deferred until other pest disciplines could be consulted on their terminology (Thill, 1993). The use of the term resistance in this document is based on the definition by Warwick (1991).

Herbicide resistance may be defined as the condition whereby a plant withstands the normal field dose of a herbicide, as a result of selection and genetic response to repeated exposure to herbicides with a similar mode of action. Susceptible plants are normally killed by recommended field doses. (Warwick, 1991, page 95).

LeBaron and McFarland (1990) have compiled some characteristics of herbicides that predispose weeds to develop resistance. Herbicides that target a single site of action; those that effectively control a wide range of weed species; soil-applied herbicides with residual activity that control germinating weeds throughout the growth season, or are applied several times per season and; recurrent applications over several seasons without rotating, alternating or combining with other types of herbicides.

Although herbicide resistance has an economic cost because of a requirement for alternative methods of control, there have also been benefits. The study of herbicide resistant weeds has expanded our knowledge of many aspects of plant biology and herbicide mode of action (LeBaron and McFarland, 1990). They are a source of genes for breeding and genetically engineering herbicide resistant crops (Mazur and Falco, 1989). In addition, there has been considerable interest in the use of herbicide resistance as selectable markers in plant research (Mazur and Falco, 1989).

Paraquat Resistance

Herbicide resistant weeds tend to arise in situations where a single herbicide exerts a considerable selection pressure. This is characteristic of herbicides with prolonged residual activity. The residual action provides the selection pressure which allows the mutant plants to thrive and reproduce. Paraquat is one of the exceptions. It is a contact herbicide that is applied to the foliage. It is inactive in soil because it becomes strongly adsorbed to clay colloids and organic matter (Brian et al., 1958). Paraquat-resistant weeds have arisen at sites where there have been multiple applications per year for many years or where paraquat was the only herbicide used for a prolonged period (Table 1). Based on the criteria of LeBaron and McFarland (1990) paraquat has a high risk for promoting the development of weed resistance. Currently, eleven species of paraquat-resistant weeds are reported to have been selected under such conditions. Although in some situations *Lolium perenne* L. is a weed, it is also a forage crop. The resistant genotypes of this species, unlike the other species listed in Table 1, were deliberately selected.

In vitro selection for paraquat resistance has also been accomplished in a few plant species: *Chenopodium rubrum* L., *Ceratopteris richardii* Brongn. and *Nicotiana tabacum* L. (Bhargava, 1993; Carroll et al., 1988; Furusawa et al., 1984; Hughes et al., 1984). In addition, attempts have been made to genetically transform various species to increase oxidative stress resistance. This was done by incorporating additional genes encoding enzymes that scavenge oxygen radicals. Whereas the enhanced production of SOD by transgenic *Solanum tuberosum* L. protected against

Table 1. Paraquat-resistant weeds (compiled from Itoh and Matsunaka, 1990; and LeBaron and McFarland, 1990).

Scientific name	Common name	Year found	Location
<i>Arctotheca calendula</i>	Capeweed	1986	Australia
<i>Conyza bonariensis</i>	Hairy fleabane	1979 1984	Egypt Hungary
<i>C. canadensis</i> <i>Erigeron canadensis</i>	Canadian fleabane	1982	Japan Hungary
<i>C. philadelphicus</i> <i>E. philadelphicus</i>	Philadelphia fleabane	1980	Japan
<i>C. sumatrensis</i> <i>E. sumatrensis</i>	Oharechinoqiku	1987	Japan
<i>Epilobium ciliatum</i>	American willowherb	1984	Belgium
<i>Hordeum glaucum</i>	Barley grass	1983	Australia
<i>H. leporinum</i>	Hare barley	1989	Australia
<i>Lolium perenne</i>	perennial ryegrass	1976	United Kingdom
<i>Poa annua</i>	Annual bluegrass	1978	United Kingdom
<i>Solanum americanum</i>	American black nightshade	1990	USA
<i>Youngia japonica</i>	Asiatic hawksbeard	1987	Japan

paraquat toxicity, transformed *N. tabacum* and *Lycopersicon esculentum* MILL. plants with enhanced SOD levels were as sensitive to paraquat as control plants (Perl et al., 1993; Tepperman and Dunsmuir, 1990). *N. tabacum* plants transformed with

glutathione reductase genes from *Escherichia coli* were less susceptible to paraquat than control plants (Aono et al., 1991, 1993).

Mechanisms of Paraquat Resistance

A review of the mechanisms of paraquat resistance was recently published (Fuerst and Vaughn, 1990). The authors discussed five mechanisms that may impart paraquat resistance. These included altered cuticular membrane; a change at the site of action; metabolism of the herbicide to an inactive form; elevated levels of protective enzymes; and rapid sequestration of the herbicide.

Altered Cuticular Membrane

A change in the composition or thickness in the cuticular membrane may limit the penetration of the leaf surface by a xenobiotic. Although this hypothesis has been investigated in a number of weeds and species, no evidence has been provided to support this as a mechanism for resistance to paraquat. Leaves from resistant (R) and sensitive (S) plants of *Hordeum glaucum* STEUD. gave the same kinetic response for paraquat uptake, therefore, cuticular penetration was not involved (Bishop et al., 1987). Fuerst et al. (1985) observed greater cuticular penetration by paraquat in the R biotype than the S biotype of *Conyza bonariensis* (L.) CRONQ. This was apparently due to the R biotype possessing a thinner cuticular membrane (Vaughn and Fuerst, 1985). There was no difference in the short term uptake of *L. perenne* among the three biotypes examined (Harvey et al., 1978).

Mutation at Site of Action

A change at the site of action in a paraquat-resistant biotype should have the effect of reducing the efficiency of paraquat reduction and thus decreasing oxygen radical formation and toxicity. Powles and Cornic (1987) used isolated thylakoids of *H. glaucum* to determine whether there were any differences in the affinity of PSI for paraquat. They found no evidence to support this. Work with intact chloroplasts and protoplasts indicated no difference in the inhibition of carboxylation. Electron paramagnetic resonance was used in a direct analysis of the site of action in *Conyza* (Norman et al., 1993). The spectra obtained did not appear to indicate any structural differences at the site of action (Norman et al., 1993). These results are considered to support earlier findings by Fuerst et al. (1985) that equivalent amounts of oxygen uptake in both biotypes were induced by paraquat, diquat and triquat (7,8-dihydro-6H-dipyrido[1,2- α :2',1'-c][1,4]diazepinediium ion) in PSI partial reactions.

Paraquat Metabolism

Metabolism of paraquat in plants has never been reported (Summers, 1980). Very little work has been done in the area of paraquat metabolism in resistant species. When the R biotype of *Lolium* was treated with a sublethal dose of ^{14}C -paraquat, no metabolites were recovered (Harvey et al., 1978). Norman et al. (1993) found no evidence of metabolism in *C. bonariensis*.

Protective Enzymes

Protective enzymes and their products scavenge toxic oxygen species and prevent lipid peroxidation of cell membranes and oxidation of other cell constituents. To serve as a primary mechanism of resistance, elevated levels of the enzymes need to be constitutive. Because of the rapid action of paraquat, induction of protective enzymes is not considered a feasible primary mechanism of resistance (Harvey and Harper, 1982). In *Lolium*, elevated constitutive levels of SOD, catalase and peroxidase were detected in crude extracts of some R genotypes. It was proposed that resistance was conferred by the enhanced ability to scavenge O_2^- and H_2O_2 (Harper and Harvey, 1978).

Reports on the constitutive levels of protective enzymes in *C. bonariensis* have been in conflict. Whereas Shaaltiel and Gressel (1986) ascribe paraquat resistance in this species to increased amounts of stromal SOD, ascorbate peroxidase and glutathione reductase, other workers have not been successful in confirming these results (Norman et al., 1993). In addition, Vaughn et al. (1989) argue that the lack of cross-resistance to other compounds that sensitize the formation of oxygen radicals indicates that resistance is not due to enzymic protection. However, Shaaltiel's group has not only provided evidence of cross-resistance to environmental and xenobiotic oxidants, but have also demonstrated that chelators of copper and zinc, which inhibit SOD, had a synergistic effect on paraquat phytotoxicity in R and S *C. bonariensis* (Shaaltiel and Gressel, 1987a; Shaaltiel et al., 1988b). The reason for the discrepancy in the data from the two groups may lie in the growth stage of plants

used in their studies. The juvenile rosette stages of both R and S biotypes of *C. bonariensis* are more susceptible to paraquat treatment than flowering plants (Amsellem et al., 1993, Jansen et al., 1990).

Conflicting results have also been obtained for *C. canadensis* (L.) CRONQ. A Hungarian group reported that the levels of protective enzymes in cell-free extracts were lower in the resistant biotype (Pölös et al., 1988). Conversely, oxygen detoxifying activities of intact chloroplasts from *C. canadensis* were twice as high in the R biotype as in the S biotype (Itoh and Matsunaka, 1990).

There was no evidence of elevation of constitutive levels of protective enzymes in leaves of *H. glaucum* and *Arctotheca calendula* (L.) LEVYNS (Powles and Cornic, 1987; Powles and Howat, 1990). Tepperman and Dunsmuir (1990) found that transformed tobacco (*N. tabacum*) plants with as much as 50-fold normal chloroplastic SOD activity were not resistant to oxidative stress. They concluded that elevation of all three protective enzymes may be necessary to produce a resistant phenotype (Tepperman and Dunsmuir, 1990). Amplification of glutathione reductase has also been accomplished in tobacco (Aono et al., 1991, 1993). The transgenic plants were less susceptible to oxidative stress produced by paraquat and sulfur dioxide.

Paraquat Sequestration

The effect of a paraquat sequestration mechanism would be to restrict penetration of the herbicide to the site of action. Through a process of elimination it was concluded that resistance in *H. glaucum* must be due to apoplastic sequestration

(Bishop et al., 1987; Powles and Cornic, 1987). Similarly, without direct evidence, sequestration has been proposed as the mechanism of resistance in *C. bonariensis* (Fuerst et al., 1985; Norman et al., 1993). *H. leporinum* LINK unlike *H. glaucum* did not exhibit apoplastic sequestration (Preston et al., 1992).

Exclusion by Plasmalemma

A sixth mechanism not covered by Fuerst and Vaughn (1990) is the exclusion of paraquat from the symplast due to a change in the plasma membrane. DiTomaso et al. (1992b) reported decreased penetration of paraquat through the plasmalemma of the R biotype of *H. glaucum*. This contradicts the work of Powles and Cornic (1987) who determined that there was no change in the permeability of the plasmalemma or chloroplast envelope in intact protoplasts.

Translocational Mutants

Some workers have demonstrated translocational differences between paraquat-resistant and -sensitive biotypes (Bishop et al., 1987; Fuerst et al., 1985; Preston et al., 1992; Tanaka et al., 1986). Carroll et al. (1988) have termed these translocational mutants. Translocation in the R biotypes was limited or nonexistent, while extensive distribution of paraquat was observed in the S biotypes. Bishop et al. (1987) regarded these differences in translocation by *H. glaucum* biotypes to be related to their proposed sequestration mechanism. Since the R biotype of *H. leporinum* does not sequester paraquat in the cell wall, the resistance mechanism in

the two species is thought to be different (Preston et al., 1992). Fuerst and Vaughn (1990) also consider restricted mobility of paraquat in R biotypes to be due to rapid sequestration of the herbicide.

The pattern of translocation of ^{14}C -paraquat fed through the petiole was investigated in *C. bonariensis*. While there was uniform movement of paraquat throughout the lamina of the S biotype, paraquat was restricted to regions along the veins (Fuerst et al., 1985). In related species, *E. philadelphicus* L. and *E. canadensis* L., Tanaka et al. (1986) found restricted uptake through the cut ends of excised leaves and limited translocation in both R biotypes. They suggested that the difference in movement of paraquat in S and R biotypes may be correlated with the resistance phenomenon.

Paraquat-Resistant American Black Nightshade

In Florida, an increase in tomato (*L. esculentum*) production and very effective weed control on raised beds has been achieved due to a combination of polyethylene mulch and soil fumigation. These measures have little effect on weeds between beds, where additional means of control are required. Of the herbicides labelled for weed control in tomatoes in Florida, paraquat is the most widely used for weed control in row middles (Gilreath et al., 1987). Directed postemergence application minimizes crop injury. One of the disadvantages of relying on paraquat is that at least three applications per season are necessary for effective control (Gilreath and Stall, 1987).

American black nightshade (*Solanum americanum* MILL.) is a pernicious weed of tomato production in Florida. Herbicide selectivity for nightshade (*Solanum* spp.) in a tomato crop is difficult to obtain since they are both solanaceous plants with similar physiology (Stall et al., 1987; Weaver et al., 1987). In the absence of an effective control strategy, nightshade can competitively reduce tomato yields (McGiffen et al., 1992; Weaver et al., 1987).

In 1987, Gilreath and Stall reported that growers in the Immokalee-Naples area had observed a reduced efficacy of paraquat on American black nightshade. Since Youngman et al. (1979) had found that a copper chelate of D-penicillamine, with superoxide dismutase activity, inhibited paraquat toxicity in flax cotyledons; Stall et al. (1987) hypothesized that the poor control of American black nightshade by paraquat was due to copper-containing fungicides. Evidence was provided that confirmed that some fungicides/bactericides decreased American black nightshade control by paraquat (Bewick et al., 1990b). However, even in the absence of the pesticides, a biotype of nightshade collected from the Naples area was shown to be 12-fold more resistant to paraquat than a sensitive biotype (Bewick et al., 1990a).

The purpose of this study was to elucidate the primary mechanism of paraquat resistance in American black nightshade, using physiological and biochemical techniques. To this end, the following null hypothesis was proposed. Paraquat resistance in American black nightshade can be explained by a previously established mechanism. The alternative hypothesis was: paraquat resistance in this species is due to a mechanism that is unique and not previously reported.

The major objectives were two-fold. The first was to determine whether the R biotype and a S biotype differed in their ability to deal with paraquat. Specifically, the intention was to investigate whether (a) in the R biotype paraquat was prevented from penetrating to the site of action; (b) it possessed an insensitive site of action or (c) was capable of metabolizing the herbicide. The second major objective was to compare the susceptibility of the two biotypes to oxidative stress. This area of investigation was intended to (a) compare the ability of the two biotypes to generate oxygen radicals and (b) compare their defenses against oxidative stress.

CHAPTER 2 COMPARATIVE PARAQUAT UPTAKE AND COMPARTMENTALIZATION

Introduction

Paraquat is a postemergent herbicide that induces rapid desiccation (Summers, 1980). A member of the bipyridinium family, its site of action is at the $F_A F_B$ iron-sulfur protein in PSI where it oxidizes photosynthetic electron transport (Fujii et al., 1990; Parrett et al., 1989). The paraquat cation is reduced to the radical form, which is instantly reoxidized by molecular oxygen, giving rise to O_2^- (Farrington et al., 1973). The accumulation of H_2O_2 , resulting from the dismutation of O_2^- , inhibits the fixation of carbon (Kaiser, 1976, 1979; Robinson et al., 1980). However, the majority of cell damage is considered to result from the reaction of cell constituents with the $OH^\bullet$ (Dodge, 1989). The latter is generated by the iron-catalyzed Haber-Weiss reaction between O_2^- and $OH^\bullet$ (Halliwell and Gutteridge, 1989).

Six mechanisms of acquiring paraquat-resistance have been proposed:

- (1) reduced cuticular penetration,
- (2) sequestration of paraquat away from the site of action,
- (3) exclusion by the plasmalemma,
- (4) elevated levels of protective enzymes,
- (5) mutation at the site of action and

(6) paraquat metabolism (Fuerst and Vaughn, 1990; DiTomaso et al., 1992b).

Although paraquat resistance has been reported in twelve weed species, evidence has been provided in support of just three mechanisms. Elevated levels of superoxide dismutase, ascorbate peroxidase and glutathione reductase have been measured in paraquat-resistant *L. perenne*, *E. philadelphicus* and *C. bonariensis* (Harper and Harvey, 1978; Itoh and Matsunaka, 1990; Shaaltiel and Gressel, 1986; Shaaltiel et al., 1988a; Youngman and Dodge, 1981). These enzymes of the Halliwell-Asada pathway are concerned with the scavenging of toxic oxygen species.

Apoplastic sequestration was considered a plausible explanation of resistance in *C. bonariensis* and *H. glaucum* (Bishop et al., 1987; Fuerst and Vaughn, 1990; Norman et al., 1993). This may involve adsorption of the herbicide in the apoplast. Cytoplasmic accumulation in an organelle other than the chloroplast is also possible. Gradual recovery of normal fluorescence, following treatment in only the R biotype, led Lehoczki and Szigeti (1988) to propose that resistance in *C. canadensis* was due to sequestration by an unknown mechanism.

Conflicting results were reported for paraquat penetration of the plasmalemma in paraquat-resistant *H. glaucum*. Powles and Cornic (1987) found no evidence of reduced plasmalemma permeability in the R biotype. However, DiTomaso et al. (1992b) found that paraquat resistance in *H. glaucum* was due to reduced herbicide transport across the plasma membrane. In a more recent report, it was proposed that resistance in this species was due to enhanced vacuolar sequestration of paraquat (Lasat et al., 1994).

In 1990, Bewick et al. reported that a biotype of American black nightshade was 12-fold more resistant to paraquat than a sensitive biotype. Although none of the previous research has demonstrated the involvement of the cuticle, one of the objectives of this work was to determine whether paraquat resistance in American black nightshade was due to differential cuticular penetration. In addition, the involvement of apoplastic sequestration and/or exclusion of the herbicide from the symplast in conferring resistance was investigated.

Materials and Methods

Plant Material

Seeds were collected from a sensitive (S) population and a resistant (R) population of American black nightshade in Alachua and Collier counties, respectively (Stall et al., 1987). Seedlings at the cotyledon stage were transplanted into 10 cm pots filled with a commercial potting mix (Metro-mix, Gracewood Horticultural Products, Cambridge, MA). Fertilizer and water were provided as a 1/3 strength Hoagland's solution (Hoagland and Arnon, 1950) via an automatic watering system fitted with a fertilizer injector, three times per day.

One hundred plants of each biotype were scored for sensitivity to paraquat at the three to four true leaf stage. A single fully expanded leaf of each paraquat-S plant was painted with 48 μ M paraquat (Ortho paraquat CL, Chevron Chemical Co., Ortho Division, San Francisco, CA). The R biotype was painted with paraquat concentrations from 0.7 mM to 6.3 mM. The leaves were assessed visually for injury

symptoms 24 h after painting and the most sensitive and the most resistant plants were selected for experimental use.

Leaf disks 5 mm in diameter were used in all experiments. Disks were cut from fully expanded leaves using a cork borer, taking care to avoid the midrib and main veins. The disks were rinsed three times with distilled water to clean the surfaces and to remove adhering debris from the cut edge. Disks were then allowed to float on distilled water until needed.

The specific leaf weight was determined as follows. Twenty-five leaf disks were blotted dry on filter paper and weighed. This was done on 42 sets of 25 leaf disks for each biotype prior to use in the first paraquat uptake experiment. Based on a total leaf area of 4.9 cm^2 , the specific leaf weight was expressed in units of g m^{-2} .

Paraquat Uptake

The buffer used in the assay of paraquat uptake consisted of 5 mM MES-TRIS pH 5.8, 0.2 mM CaCl_2 , 0.1% Tween 20 and $0.7 \mu\text{M}$ ($0.1 \mu\text{Ci}$) ^{14}C -paraquat. The ring-labeled paraquat was provided by ICI Agrochemicals (Jealott's Hill Research Station, Bracknell, Berkshire, UK). Three milliliters of uptake buffer were added to each 25 mL Erlenmeyer flask. Twenty-five leaf disks were blotted dry on filter paper and placed in the buffer. The flasks were placed on a rotary shaker set at 100 rpm. Time dependent uptake was performed for 0, 5, 10, 20, 30, 45, 60, 75, 90 and 180 minutes. A $50 \mu\text{L}$ aliquot of the buffer was sampled prior to addition of the disks and at the end of the uptake period. Aliquots were placed in 20 mL liquid scintillation

vials. Ten milliliters of Aquasol universal liquid scintillation fluid (E.I. du Pont de Nemours & Co., Boston, MA) were dispensed to each vial. The number of disintegrations per minute (dpm) were determined by liquid scintillation spectrometry (LSS) using a Beckman LS 7500 Liquid Scintillation System (Beckman Instruments Inc., Fullerton, CA). Paraquat absorbed by the leaf disks was calculated as the difference between the measurements taken before and after the uptake period.

The treatments were replicated 3 times and the experiment was conducted three times. The data from the three experiments were combined and analyzed as a randomized complete block design. The SAS System (SAS Institute Inc., Cary, NC) was used for data analysis. Analysis of variance (ANOVA) was performed using the general linear models (GLM) procedure. The nature of the response was determined by regression analysis of the uptake means against time.

In order to clarify whether the paraquat concentration being used was injuring the leaf disks, an assay for ethane was performed on nontreated and paraquat-treated disks. After the uptake period, the leaf disks were separated from the uptake solution with a Buchner funnel and quickly rinsed with distilled water. The disks were blotted dry and placed in 21 by 70 mm glass vials. The vials were sealed with serum stoppers and illuminated for 5 min at $300 \mu\text{E m}^{-2} \text{s}^{-1}$. A 1 mL sample of the headspace was analyzed for ethane by gas chromatography using a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard Company, Santa Clara, CA) fitted with an alumina column. The temperature settings of the injector, oven and detector were 200°C, 50°C and 250°C, respectively.

Paraquat Efflux

For each biotype, 50 leaf disks were blotted dry and placed in a 25 mL Erlenmeyer flask containing 3 mL uptake buffer. A 50 μ L aliquot of the buffer was removed before and after the 30 min loading period in order to determine uptake. The flasks were allowed to shake during uptake and the subsequent efflux period at 100 rpm. The disks were separated from the uptake buffer by vacuum filtration and transferred to a clean flask. Paraquat on the surface of the leaf disks was removed by quickly rinsing with 2 mL of efflux buffer (uptake buffer minus paraquat). The flask was evacuated using a water pump and a 3 mL volume of efflux buffer was added to the flask, 1 mL of which was sampled at the end of the efflux interval. Paraquat efflux was determined after 5, 10, 15, 30, 45, 60, 90 and 120 min. The amount of ^{14} C-paraquat contained in the aliquot was determined by LSS and adjusted for a 3 mL volume.

The treatments were replicated 3 times. The experiment was conducted twice and the data were combined. A repeated-measures ANOVA was required since sequential efflux measurements were done over time on the same leaf disks. The analysis was performed using a split-plot approach. This allowed for a more stringent hypothesis test of the main effect of biotype.

Paraquat Compartmentalization

A difference between the biotypes in the efflux of paraquat could have been due to adsorption within the apoplast or differential uptake into the symplast. To

distinguish between these possibilities an experiment was conducted to characterize paraquat compartmentalization. Short-term efflux over a 15 min period was determined in order to compare the amount of paraquat present in an unbound state in the apoplast. The loading period was performed as described in the previous section. Instead of rinsing, paraquat on the surface of the leaf disks was removed by blotting with filter paper. Efflux was also performed similarly, except that the efflux period was restricted to 15 min.

On completion of efflux of unbound apoplastic paraquat, another 3 mL volume of efflux buffer was added. The plasma membranes of the leaf disks were then disrupted by two cycles of freezing and thawing. The freezing was done by partially immersing the flasks in liquid nitrogen. The buffer was thawed at room temperature. The flasks were then allowed to shake for 15 min to promote maximum release of cytoplasmic paraquat. A 1 mL aliquot was sampled for LSS and the flask evacuated.

Finally, the tissue was exposed to 3 mL of desorption buffer (5 mM MES-TRIS pH 5.8 and 5 mM CaCl_2) for 15 min in order to displace bound apoplastic paraquat. A 1 mL aliquot was sampled and subjected to LSS.

Silanization of Glassware

During preliminary uptake experiments it became apparent that ^{14}C -paraquat was being lost from the uptake buffer which could not be explained by uptake into the leaf tissue. A desorption procedure with 0.5 M NaCl solution revealed that paraquat was being adsorbed to the glass surface of the Erlenmeyer flask. In order to prevent

this from occurring, the glassware was siliconized using 5% dichlorodimethylsilane in toluene in a chemical hood. After evaporation of the toluene, the glassware was baked for 2 h at 180°C or rinsed thoroughly with water before use (Sambrook et al., 1989).

Results

Paraquat Uptake

The paraquat uptake study was conducted in order to determine whether differences in cuticular penetration was the mechanism of paraquat resistance in *S. americanum*. The biotypes did not differ in their rate of paraquat absorption (Fig. 5). Therefore, the cuticular membrane was not a barrier to uptake in the R biotype and does not appear to be involved in conferring resistance. Time-dependent paraquat uptake in *S. americanum* was best described by a quadratic relationship. Although the S biotype had a greater mass per unit leaf area (Table 2), this did not appear to influence paraquat uptake. Ethane evolution by the leaf disks was measured in order to determine whether membrane damage occurred during the course of uptake. There was no difference ($p > 0.05$) between the amount of ethane evolved by untreated and paraquat-treated disks over the 3 h uptake period (Fig. 6). In addition, no biotypic difference was observed. Therefore, the $0.7 \mu\text{M } ^{14}\text{C}$ -paraquat did not cause detectable membrane damage in either biotype over the course of the experiments.

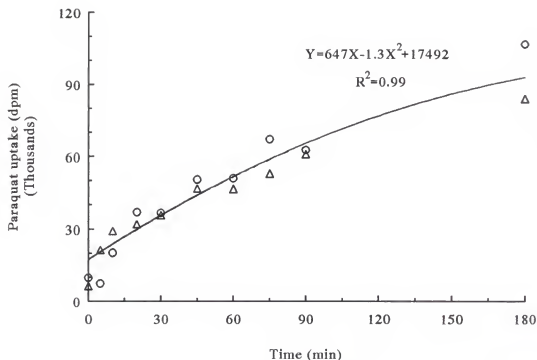


Figure 5. Time-dependent ^{14}C -paraquat uptake in *S. americanum*. The markers indicate the means of 3 experiments. The paraquat-S biotype is designated by (Δ) and paraquat-R biotype by ($\circ$).

Table 2. Specific leaf weight of paraquat-resistant and -sensitive American black nightshade. Twenty-five 5 mm leaf disks were weighed. There were 42 replicates per biotype.

Biotype	Specific leaf weight (g m^{-2})
Sensitive	157.6 (2.2) ^a
Resistant	148.8 (1.2)
LSD	5.0

^aFigures in brackets are standard errors of the mean.

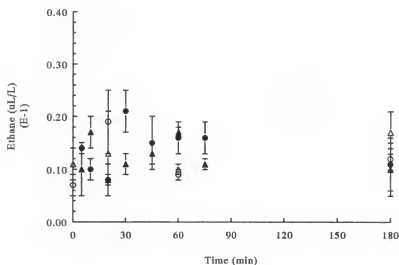


Figure 6. Comparison of ethane evolution from nontreated S biotype (Δ) and ($\circ$), and paraquat-treated ($0.7 \mu\text{M}$) S biotype ($\blacktriangle$) and R biotype ($\bullet$).

Paraquat Efflux

Paraquat efflux studies were used to characterize paraquat uptake and distribution. Disks were allowed to load paraquat for 30 min prior to determining efflux. Efflux data were expressed as a percentage of absorbed paraquat. Two phases of efflux were observed: a rapid phase occurring during the first 10 min, followed by a slower phase (Fig. 7). The rapid phase is thought to be due to efflux from the apoplast, and the slower phase to be due to efflux from the symplast (Nandihalli and Bhomik, 1991; Van Ellis and Shaner, 1988). Over the 2 h efflux period, more paraquat effluxed from the S biotype than the R biotype. There was a significant interaction between biotype and time ($P < 0.05$) indicating that the rate of paraquat efflux was not the same. This pattern of efflux could be due to greater

adsorption in the apoplast or increased uptake into the symplast by the R biotype.

Therefore, compartmentalization studies were conducted to determine the distribution of paraquat after a loading period of 30 min.

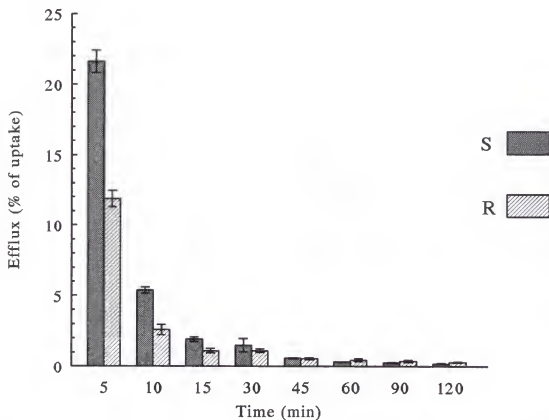


Figure 7. Time-course comparing the efflux of ^{14}C -paraquat from paraquat-sensitive (S) and -resistant (R) biotypes of American black nightshade.

Paraquat Compartmentalization

As for paraquat efflux, the data from the compartmentalization study also were expressed as a percent of paraquat uptake (Table 3). The paraquat measured during short-term efflux constituted the unbound apoplastic component. The total amount of

cell-free paraquat of the S biotype exceeded that of the R biotype. The rate of efflux of paraquat was faster in the S biotype. Although more paraquat was adsorbed by the R biotype than the S biotype, there was no difference in the percentage of absorbed paraquat that penetrated into the cytoplasm. This indicated that it was unlikely that paraquat resistance in American black nightshade was due to apoplastic sequestration or to exclusion by a less permeable plasmalemma.

Table 3. Compartmentalization of ^{14}C -paraquat. Free apoplastic paraquat was determined by short-term efflux, bound apoplastic paraquat by desorption and cytoplasmic paraquat by membrane disruption.

Treatment	Biotype		LSD
	Sensitive	Resistant	
	dpm		
Uptake	52284 (2567)	65429 (5801)	13815
Efflux (min)	% of uptake		
2.5	24.4 (0.7)	15.6 (1.3)	
5.0	5.8 (0.3)	3.4 (0.2)	
7.5	3.2 (0.2)	1.5 (0.1)	
10.0	1.8 (0.1)	0.8 (0.1)	
15.0	1.8 (0.2)	0.8 (0.1)	
Total efflux	37.0 (1.0)	22.2 (1.2)	3.6
Desorption	11.9 (0.6)	14.9 (0.8)	2.6
Freeze/thaw	40.0 (4.5)	47.6 (4.5)	9.6
Total	88.9 (5.0)	84.7 (5.3)	14.5

Discussion

Initially, the intention was to study paraquat penetration through isolated cuticular membranes. However, enzymic removal of the cuticular membranes could

not be consistently performed with *S. americanum*. When isolations were successful, the cuticles proved to be too fragile to withstand subsequent procedures. The use of leaf disks was a compromise. It allowed the assessment of paraquat uptake but did not distinguish between cuticular penetration and plasmalemma penetration. Other studies of paraquat uptake in resistant weeds have utilized intact attached leaves (Bishop et al., 1987) and detached leaves, the petioles of which were maintained in water (Fuerst et al., 1985). In the first case, differing rates of translocation of paraquat away from the uptake site may have masked differences in cuticular penetration. In both cases, the authors claimed to have determined the amount of paraquat that partitioned into the cuticle by means of chloroform washes. This technique will estimate only the fraction that is found in the epicuticular waxes and not the entire cuticle (Bewick et al., 1993). Only negligible amounts of paraquat were retrieved, and did not correlate with resistance (Bishop et al., 1987; Fuerst et al., 1985).

In concurrence with our findings, paraquat uptake through the cuticular membrane was not correlated with paraquat resistance in *C. bonariensis*, *H. glaucum* or *L. perenne* (Fuerst et al., 1985; Bishop et al., 1987; Harvey et al., 1978; Youngman and Dodge, 1981). In *H. glaucum* and *L. perenne*, no difference in uptake was observed (Bishop et al., 1987; Harvey et al. 1978). However, Fuerst et al. (1985) reported greater cuticular penetration in the R biotype of hairy fleabane while Youngman and Dodge (1981) found no biotypic difference in uptake in the same species.

The concentration of paraquat in the solutions used in these studies was restricted to a relatively low level because 8.5 μM paraquat was observed to cause membrane damage in both biotypes within 2 h (Chapter 3). More extensive characterization of uptake and compartmentalization has been accomplished with maize roots (DiTomaso et al., 1993; Hart et al., 1992a). These authors had much more flexibility in the concentrations and time courses they could utilize because root tissue lacks chloroplasts and the phytotoxic action of paraquat is much slower. Such a system was not practical in this situation since the effect of the cuticular membrane was of interest.

Examination of the kinetics of paraquat uptake across the plasmalemma in maize roots revealed a saturable component, which indicated a protein-mediated system (Hart et al., 1992a). Further, uptake apparently occurred via the putrescine membrane carrier system (Hart et al., 1992b). Paraquat uptake by plant cells is thought to occur by passive transport (facilitated diffusion), the driving force being its electrochemical gradient across the plasmalemma (Hart et al., 1993). This has been corroborated by Zer et al. (1993), using a suspension of dark-grown *Phaseolus vulgaris* L. cells, who found that spermine competitively inhibited the uptake of paraquat. Uptake of paraquat was also reduced by low temperature and by the addition of an uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)¹ (Zer et al., 1993).

¹CCCP is a proton-specific ionophore which is used to disrupt the pH gradient across the plasmalemma.

Reduced paraquat uptake in an R biotype of *H. glaucum* was interpreted as being due to a change in the putrescine membrane carrier system (DiTomaso et al., 1992a). The involvement of apoplastic sequestration or binding to the cell membranes were eliminated as possibilities in these studies. This contradicted an earlier report that showed no difference in carbon dioxide fixation by S and R protoplasts, indicating that the resistance mechanism was not differential plasmalemma permeability (Powles and Cornic, 1987). More recently, based on a study of paraquat uptake into plasmalemma vesicles isolated from the leaves of two *H. glaucum* biotypes, Hart and DiTomaso (1994) have revised their earlier position. Data derived from investigations of paraquat uptake by *H. glaucum* roots was interpreted as showing no dramatic differences between the two biotypes. Instead, it was proposed that paraquat resistance is due to enhanced sequestration of the herbicide in the vacuole by the R biotype (Lasat et al., 1994).

By a process of elimination of other mechanisms, some groups have concluded that the R biotype must be sequestering paraquat (Fuerst et al., 1985; Norman et al., 1993; Powles and Cornic, 1987; Lehoczki and Szigeti, 1988). No direct evidence was provided to demonstrate paraquat binding in the apoplast or accumulation in an organelle or vacuole. On the contrary, the lack of binding to insoluble cell wall fragments observed by Fuerst et al. (1985) was later reinterpreted by Norman et al. (1993) to perhaps be due to a requirement for a structurally intact cell wall.

In summary, neither the cuticle nor the plasmalemma was a barrier to the penetration of paraquat into the cytoplasm of *S. americanum*. It is unlikely that the

primary mechanism of paraquat resistance is due to apoplastic sequestration, because three-fold more paraquat was detected in the cytoplasm than was adsorbed in the apoplast of the R biotype by the end of the 30 min loading period. In addition, there was no biotypic difference in the percentage of cytoplasmic paraquat. These results suggest that the resistance mechanism is located in the cytoplasm. These techniques did not eliminate the involvement of symplastic sequestration. Therefore, the possibility of paraquat resistance being due to restricted penetration to the site of action will be addressed in the next chapter.

CHAPTER 3

DIFFERENTIAL BIOCHEMICAL RESPONSES OF RESISTANT AND SENSITIVE BIOTYPES OF AMERICAN BLACK NIGHTSHADE TO BIPYRIDINIUM HERBICIDES

Introduction

In the previous chapter, it was shown that the S and the R biotypes of American black nightshade accumulated similar amounts of paraquat in the symplast. A number of possibilities exist that may explain resistance occurring at the cytoplasmic level. If the R biotype were capable of rapid sequestration of paraquat in the vacuole or some other organelle, this could drastically reduce the amount that was available for penetration into the chloroplast. This would result in reduced oxidative stress. Rapid metabolism of the herbicide is unlikely since paraquat metabolism has never been reported (Norman et al., 1993). In the absence of sequestration and metabolism, it is also possible that the site of action in PSI may have undergone mutation resulting in less efficient paraquat reduction. No evidence has thus far been provided in support of this mechanism. Paraquat resistance can also be conferred by elevated levels of protective enzymes (Harper and Harvey, 1978; Itoh and Matsunaka, 1990; Shaaltiel and Gressel, 1986).

Paraquat toxicity is mediated by oxygen radicals (Farrington et al., 1977). Oxygen radicals are capable of initiating and propagating the peroxidation of cell

membrane lipids (Halliwell and Gutteridge, 1989). Consequently, techniques that can quantify membrane damage occurring in response to paraquat application can be useful indicators of whether paraquat is penetrating to the site of action and exhibiting activity. Riely et al. (1974) found that the production of lipid peroxides and ethane evolution by mammalian tissue were closely correlated and proposed that *in vivo* lipid peroxidation could be assessed by measuring ethane evolution. Ethane evolution has also been demonstrated in plant tissue exposed to conditions favoring peroxidation (Konze and Elstner, 1978). The detection of ethane is regarded as a useful means of characterizing the activities of some herbicides, if the results can be corroborated by other methods of measuring lipid peroxidation (Elstner and Pils, 1979). Ethane measurement has been used in the assessment of diphenyl ether and paraquat toxicity as well as the effect of the fungal toxin cercosporin on tobacco membrane lipids (Daub, 1982; Finckh and Kunert, 1985; Kunert and Böger, 1984; Youngman et al. 1979).

The measurement of electrolyte leakage is another common method of detecting lipid membrane damage (Whitlow et al., 1992). When plant tissue is exposed to conditions that cause membrane injury, the ability of the plasma membrane to regulate the transport of ions is impaired. Leakage of electrolytes from the tissue occurs, which can be detected by measuring the conductance of the bathing solution.

Prior to Munday and Govindjee (1969), penetration of intact cells by paraquat had not been demonstrated. These authors showed that after adding paraquat to algal cells, there was a progressive quenching of peak fluorescence (F_p) over 15 minutes,

which proved that the herbicide penetrated to the site of action. In paraquat-resistant plants chlorophyll fluorescence induction measurement has been used to study the presence and activity of paraquat at its site of action in the chloroplast (Fuerst et al., 1985; Lehoczki et al., 1992; Vaughn et al., 1989).

The objectives of this study were to investigate whether paraquat can penetrate to the site of action in the R biotype, and to evaluate the biochemical responses of a paraquat-S and -R biotypes of American black nightshade to diquat and paraquat. This was accomplished indirectly through the detection of ethane and ion leakage and directly by measurement of chlorophyll *a* fluorescence.

Materials and Methods

Ethane Assay

Leaf disks, 13 mm in diameter, were cut with a cork borer and held in water to prevent wilting. The disks were rinsed three times with deionized water before use. Ten leaf disks were placed in 25 ml Erlenmeyer flask containing 8 ml of incubation solution. The incubation solutions contained 5 mM MES pH 5.2, 1 mM KCN and paraquat at one of three rates: 0, 8.5 and 85 μM . The KCN was included to inhibit the protective enzymes of the Halliwell-Asada pathway. The flasks were foil-wrapped in order to exclude light. The leaf disks were vacuum infiltrated with the incubation solution and the flasks were sealed with rubber septum stoppers.

The leaf disks were allowed to absorb paraquat in darkness for 2 h. The foil was then removed and the disks exposed to light at 300-400 $\mu\text{E m}^{-2}\text{s}^{-1}$. The light was

provided by 3 incandescent fixtures and was filtered through a 0.2 M CuSO_4 solution to reduce heat. The disks were illuminated for 2, 4, 6, and 8 h. Each combination of paraquat and period of illumination was replicated 3 times.

The headspace of each flask was mixed by repeatedly filling and releasing a 1 ml tuberculin syringe equipped with a sub-Q 26G 16 mm needle (outside diameter = 0.46 mm). A 1 mL aliquot of the headspace gas was removed and assayed for ethane using a Hewlett-Packard HP5710A gas chromatograph equipped with a flame ionization detector and a packed alumina column. The oven temperature was set at 100°C. Ethane was quantified by comparison with a 10 $\mu\text{L L}^{-1}$ standard (Scott Specialty Gases, Inc., Plumsteadville, PA). The experiment was repeated and the data from 2 experiments were combined. The data were analyzed as a randomized complete block design. The procedure used was analysis of variance (ANOVA). Means were generated and are presented with standard errors.

Ion Leakage

Plants of the S and R biotypes were scored (as described previously) in order to select plants that were most sensitive and most resistant to paraquat. Several different groups of plants were used in the performance of these studies and the relative resistance and sensitivity varied from group to group. Leaf disks were cut to give a diameter of 10 mm. The disks were washed three times with distilled water and floated on distilled water until required.

These experiments were performed using scintillation vials that were silanized to prevent adsorption of paraquat to the glass. A preliminary experiment was conducted to compare the time-dependent effect of a lethal dose of paraquat on ion leakage from leaf disks incubated in light ($300 \mu\text{E m}^{-2}\text{s}^{-1}$) and in darkness. Time-courses of 36 h for the illuminated incubations and 48 h for the dark incubations were used. The dark treatments were done at room temperature (26°C). The temperature of the illuminated treatments was maintained at 26°C , by immersing the vials in a water bath and by using a water filter to reduce infrared radiation from the lamps. Five leaf disks were placed in 5 mL aliquots of 0.3 M paraquat solution.

The initial conductivity of the paraquat solutions was determined prior to the addition of the leaf disks using a YSI Model 31 Conductivity Bridge (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio). Final conductivity was measured at the end of the incubation period, and total conductivity measured after 2 cycles of alternate freezing and thawing. Ion leakage was expressed as a percentage of the total conductivity.

Concentration-dependent assays were also conducted. In the first experiment the disks were allowed to incubate in light ($300 \mu\text{E m}^{-2}\text{s}^{-1}$) for 8 h or darkness for 12 h at 26°C . The paraquat concentrations ranged from 0 to 0.43 mM for the S biotype and 0 to 8.5 mM for the R biotype. In the next 2 experiments, the concentration ranges were reduced to 0 to 17 μM for the S biotype and the upper limit for the R biotype was 2.1 mM. The incubation times were extended to 12 h in the light and to 24 h for dark incubation. In all 3 experiments the treatments were replicated 3 times.

The data were sorted by illumination type and biotype. The raw data as well as treatment means were analyzed using the REG procedure of SAS. I_{50} values (the paraquat concentration required to cause loss of 50% of the electrolytes) were determined. I_{50} values and 95% confidence limits were calculated using inverse regression for a linear model. Where the model was not linear, the data were first transformed using a square root or natural log transformation.

Chlorophyll *a* Fluorescence

Chlorophyll *a* fluorescence was measured using a Plant Productivity Fluorometer Model SF-20 (Richard Brankner Research Ltd, Ottawa, Canada). The experiments were done with 10 mm leaf disks prepared as described previously. The leaf disks were floated on a dish of distilled water and held in darkness for 1 h. The experiments were conducted in a darkroom using a green safelight. After dark-adaption, the leaf disks were blotted dry with filter paper. Disks were placed adaxial side down in 15 mL of 20 μ M diquat or 1.7 mM paraquat in plastic Petri plates. The latter were covered with foil to exclude light. Leaf disks were allowed to incubate for 10 to 60 min. At the end of the incubation period, disks were removed from the herbicide solution and placed adaxial side down on the sensing probe of the fluorometer. Initial, peak and terminal fluorescence were measured. These terms correspond to F_0 , F_p and F_s using the nomenclature of van Kooten and Snel (1990). F_s was measured 50 s after F_0 . Variable fluorescence (F_v) was determined by taking the difference between F_p and F_0 . Data for a single diquat experiment and 2 paraquat

experiments were used in the respective analyses. The experiments were analyzed using the ANOVA procedure of SAS. The experimental designs were randomized complete block.

Concentration-dependent fluorescence was also measured. The dark-adaptation was combined with herbicide incubation. In the preliminary experiment, which included both diquat and paraquat, the dark-adaptation/incubation period was 30 min. The herbicide concentrations ranged from 0 to 20 mM for diquat and 0 to 17 mM for paraquat. It was intended that the same concentrations be used for both herbicides, however, the hydration of paraquat was inadvertently not considered when the solutions were prepared. Concentration-dependent experiments were repeated 2 more times using paraquat only and an incubation period of 20 min. An additional concentration of 170 mM was added for the R biotype only. The experiments were analyzed as randomized complete block designs using the GLM procedure of SAS. The analysis was adjusted to accommodate the unequal number of treatments used in the last concentration-dependent experiment. This required using the type IV sums of squares for hypothesis testing (Littel et al., 1992).

Results

Ethane Evolution

Biotypes differed in the amount of ethane they produced (Fig. 8). However, the response was not the same over all levels of time and paraquat concentration. Since paraquat was expected to cause membrane injury, the data were sorted by

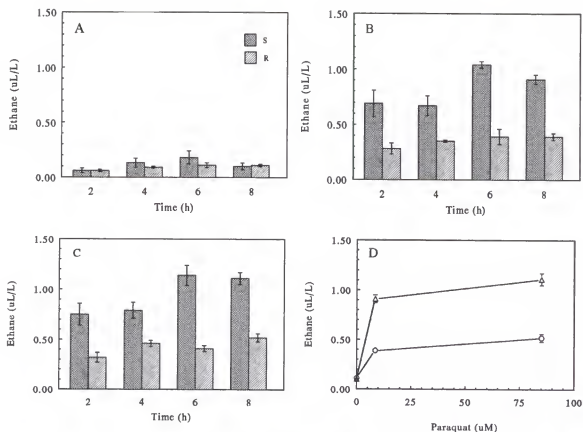


Figure 8. Ethane evolution over time by leaf disks treated with 0 (A), 8.5 (B) and 85 μM (C). Paraquat-dependent ethane production by the S biotype (Δ) and the R biotype ($\circ$) is shown in (D).

paraquat concentration and reanalyzed. The nontreated (0 paraquat) leaf disks evolved ethane over time (Fig. 8a). This was expected since inefficiencies in photosynthetic electron transport chain result in the reduction of O_2 to form O_2^- (Asada et al., 1974). The addition of KCN was intended to inhibit the scavenging of O_2^- , so that possible differences in constitutive levels of protective enzymes would not confound the results. Although there was a tendency for the S biotype to produce more ethane than the R biotype, this was not significant (Fig. 8a). The addition of paraquat resulted in a considerable increase in ethane production. There was a

significant interaction ($P < 0.05$) between biotype and time for the 8.5 and the 85 μM concentrations. This was due to a more rapid rate of ethane evolution by the S biotype (Fig. 8b,c). Increasing the concentration of paraquat from 8.5 to 85 μM resulted in a small but significant ($P < 0.05$) increase of ethane production by both biotypes (Fig. 8d). These results indicate that paraquat was capable of penetrating the chloroplasts of the R biotype and generating oxidative stress. However, membrane damage occurred more rapidly in the S biotype than in the R biotypes.

Electrolyte Leakage

There was no biotypic difference in the amount of ion leakage in the absence of paraquat (Fig. 9a). Ion leakage increased with time of incubation in both darkness and light. However, in the former case there was a biotype by time interaction ($p < 0.05$) which indicated that the slope of the response was unique for each biotype. The time-dependent response was the same for both biotypes in the light. The addition of paraquat increased ion leakage significantly (Fig 9b). Whereas untreated leaf disks leaked more ions in the darkness than in the light, the reverse was true for paraquat-treated disks. Ion leakage occurred more rapidly under illuminated conditions when paraquat was present. This was expected since bipyridyl toxicity is accelerated due to oxygen radical production resulting from paraquat photoreduction in the chloroplast (Mees, 1960). There was a significant interaction ($p < 0.05$) between biotype and time which was indicative of differences in biotypic response over time. Further analysis indicated that the interaction was due to a lower amount

of ion leakage recorded for the R biotype at 24 h than for the S biotype (Fig. 9b).

The S biotype had a more rapid rate of ion leakage than the R biotype when incubated in light.

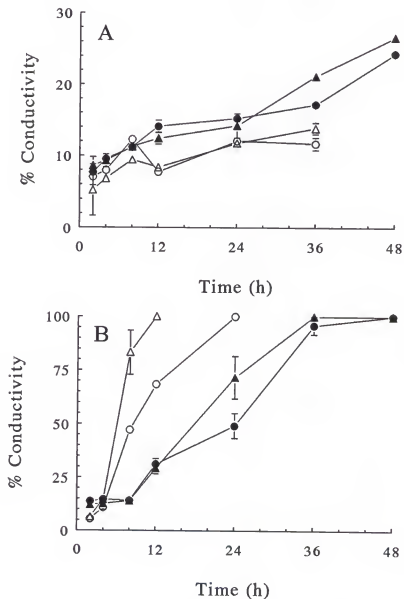


Figure 9. Time-course of ion leakage from untreated leaf disks (A) or leaf disks incubated in 0.3 mM paraquat (B). R and S biotypes incubated in darkness are depicted by ● and ▲, and in light ○ and △, respectively.

Concentration-dependent experiments were also performed. The data for these experiments (Fig. 10, 11 and 12) were not combined since incubation times of the first experiment were not consistent with those of the other two experiments, and the paraquat concentrations used for the light-incubations of the S biotype were different in each experiment. The regressions of ion leakage on paraquat concentration were performed using means of the three replicate treatments. The equations and response curves for the effect of paraquat concentration on ion leakage are illustrated in Figures 10, 11 and 12. Within the concentration ranges used, both biotypes showed an increase in ion leakage with increasing rates of paraquat in darkness and in light. In experiments 1 and 2, dark-incubated leaf disks of the R biotype had lower levels of ion leakage than those of the S biotype (Fig. 10a, 11a). There was no biotypic difference in ion leakage in experiment 3 (Fig. 12a). Whereas the difference in ion leakage between biotypes in darkness was small or absent, in light, the difference was very large. This necessitated the use of lower paraquat concentrations in order to characterize the response in the S biotype and precluded the use of ANOVA to compare the 2 biotypes in light. However, the greater sensitivity of the S biotype in the light was clearly apparent when the I_{50} values for the two biotypes were compared (Table 4). The parameter estimates that were used to calculate the I_{50} values and 95% confidence limits were derived from regression analysis of the raw data rather than means since the resulting confidence limits are more sensitive. The parameter estimates and the R^2 for the regression model were tabulated (Table 5). Ratios of I_{50} (dark): I_{50} (light) were calculated for each biotype (Table 4). The ratios ranged from

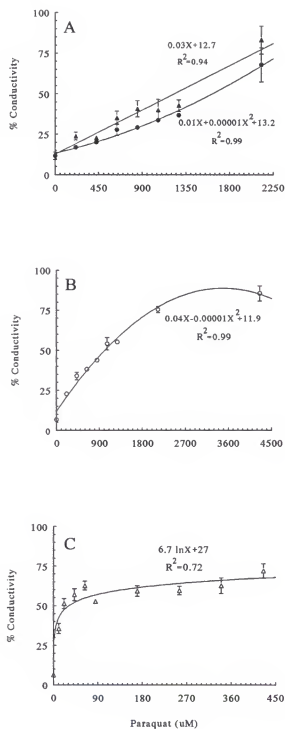


Figure 10. Experiment 1: Concentration-dependent ion leakage in darkness for 12 h by leaf disks of the R (●) and S (▲) biotypes (A) and in light for 8 h by the R biotype (○) (B) and the S biotype (Δ) (C).

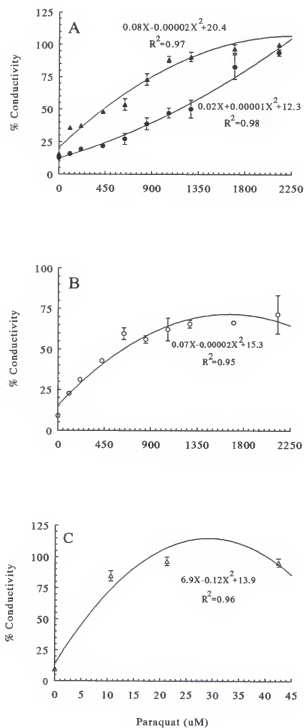


Figure 11. Experiment 2: Concentration-dependent ion leakage in darkness for 24 h by leaf disks of the R [●] and S [▲] biotypes (A), and in light for 12 h by the R biotype [○] (B) and the S biotype [△] (C).

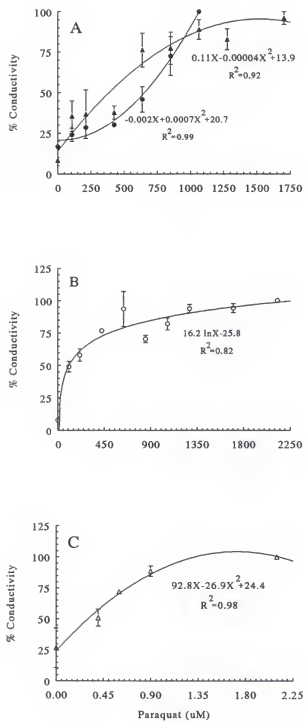


Figure 12. Experiment 3: Concentration-dependent ion leakage in darkness for 24 h by leaf disks of the R [●] and S [▲] biotypes (A), and in light for 12 h by the R biotype [○] (B) and the S biotype [△] (C).

Table 4. Estimates of I_{50} ^a values and ratios of $I_{50}(\text{dark}):I_{50}(\text{light})$ derived from the concentration-dependent electrolyte leakage studies.

	Biotype			
	Sensitive		Resistant	
	Dark	Light	Dark	Light
Expt 1	μM			
I_{50}	1230	31.3	1610	1079
Lower 95 % CI	1100	14.4	1449	971
Upper 95 % CI	1391	50.3	1824	1198
Dark/Light	39.3		1.5	
Expt 2				
I_{50}	490	4.1	1071	700
Lower 95 % CI	410	0.7	991	586
Upper 95 % CI	562	8.1	1157	827
Dark/Light	119.5		1.5	
Expt 3				
I_{50}	353	0.31	534	111
Lower 95 % CI	247	0.18	454	98
Upper 95 % CI	460	0.42	619	121
Dark/Light	1138.7		4.8	

^a I_{50} refers to the concentration of paraquat required to cause the leakage of 50% of the electrolytes from the leaf disks.

1.5 to 4.8 for the R biotype and from 39 to 1139 for the S biotype. The discrepancy in the I_{50} values between the biotypes can be explained by a mechanism of paraquat resistance that is light-dependent. There are three possible explanations for the variability of the I_{50} values within biotypes, particularly the S biotype. The most

Table 5. Parameter estimates used in the determination of I_{50} values and confidence limits, derived from regression of percent conductivity (Y) against paraquat concentration (X).

Biotype	Illumination	Intercept	X	ln X	sqrt X	R ²
Expt 1						
S	Dark	12.7	0.03	-	-	0.85
S	Light	27.0	-	6.7	-	0.57
R	Dark	9.8	0.03	-	-	0.87
R	Light	7.3	-	-	1.3	0.95
Expt 2						
S	Dark	10.9	-	-	2.1	0.93
S	Light	21.9	-	-	13.8	0.82
R	Dark	8.0	0.04	-	-	0.92
R	Light	12.0	-	-	1.4	0.85
Expt 3						
S	Dark	8.0	-	-	2.2	0.79
S	Light	83.5	-	28.5	-	0.82
R	Dark	11.1	0.07	-	-	0.83
R	Light	-26.6	-	16.3	-	0.76

sensitive response to paraquat by the S biotype apparently occurs after an incubation period greater than the 8 h used for the light incubation in experiment 1. In addition, different groups of plants were used for each experiment and so different growth conditions may have influenced the susceptibility to paraquat. It is also possible that the inherent genetic variability of the S biotype may be greater than that of the R biotype since the S biotype had not been subjected to the selection pressure of

multiple paraquat sprays per season for several years. Both the time-dependent and concentration-dependent experiments gave evidence of paraquat activity in darkness and in light. However, the biotypic difference in ion leakage was greater in the light than in darkness.

Chlorophyll A Fluorescence

Time-dependent effects of diquat and paraquat

No biotypic difference in initial fluorescence (F_0) was observed when leaf disks were incubated in 20 μ M diquat (Table 6). Although F_0 appeared to decrease with time, this was not significant. Peak fluorescence (F_p) was rapidly quenched in the presence of diquat so that, after an hour, F_p had been reduced to about one half the level of untreated plants. Quenching of F_p occurred to the same extent in both biotypes. Similarly, there was no biotypic difference in the time-dependent response of terminal fluorescence (F_s). An equivalent decrease in F_s was observed in both S and R resistant biotypes (Table 6).

Variable fluorescence (F_v) is a frequently used parameter in the early detection of stress in plants (Lichtenthaler, 1988). The level of F_0 is directly related to the chlorophyll concentration per unit leaf area (Lichtenthaler, 1988). Therefore, F_v is more useful than F_p since it adjusts for differences in chlorophyll content. The time-dependent effect of diquat on F_v was summarized in Figure 13. The longer the incubation period in the diquat solution, the greater was the reduction of F_v in both biotypes. F_v was reduced to a lower level in the S biotype (Table 7). Although there

was no significant difference in F_p and F_o , the small differences that did occur combined to cause the biotypic difference in F_v . The absence of an interaction between biotype and time means that the suppression of fluorescence occurred at the same rate and provides evidence of similar rates of diquat penetration to the site of action.

Table 6. Time-dependent effect of 20 μ M diquat on initial, peak and terminal fluorescence of leaf disks from paraquat-sensitive and -resistant biotypes.

Time (min)	Relative Chlorophyll Fluorescence					
	Sensitive			Resistant		
	F_o	F_p	F_s	F_o	F_p	F_s
0	57.7 $\pm$ 1.5	126.0 $\pm$ 4.6	72.7 $\pm$ 4.4	57.7 $\pm$ 1.9	134.3 $\pm$ 4.5	74.0 $\pm$ 1.5
10	58.7 $\pm$ 3.3	92.7 $\pm$ 3.0	70.0 $\pm$ 1.5	51.3 $\pm$ 5.9	99.3 $\pm$ 12.7	64.0 $\pm$ 6.4
20	52.3 $\pm$ 1.7	69.3 $\pm$ 3.9	63.7 $\pm$ 1.8	47.7 $\pm$ 6.4	74.3 $\pm$ 10.2	62.0 $\pm$ 5.9
30	52.0 $\pm$ 3.5	67.3 $\pm$ 2.2	61.7 $\pm$ 2.4	52.0 $\pm$ 1.2	73.3 $\pm$ 4.2	64.3 $\pm$ 2.0
40	44.7 $\pm$ 2.6	57.0 $\pm$ 1.7	56.0 $\pm$ 2.6	49.7 $\pm$ 1.8	66.7 $\pm$ 5.0	62.7 $\pm$ 2.6
50	50.7 $\pm$ 2.8	65.7 $\pm$ 4.1	62.7 $\pm$ 2.8	53.0 $\pm$ 1.0	72.0 $\pm$ 0.6	66.3 $\pm$ 1.3
60	52.7 $\pm$ 0.7	65.7 $\pm$ 2.7	65.3 $\pm$ 1.3	50.3 $\pm$ 2.0	64.3 $\pm$ 1.8	61.3 $\pm$ 1.8

When the effect of incubating leaf disks in 1.7 mM paraquat was examined over a 60 minute time-course, there was no biotypic difference in F_o (Table 8). However, there was a decrease in F_p as time of exposure to the herbicide increased. Unlike diquat, paraquat suppressed F_p to a greater extent in the S biotype than the R biotype. F_s was observed to decrease over the time-course with no biotypic difference (Table 8). There was greater inhibition of fluorescence in the S biotype

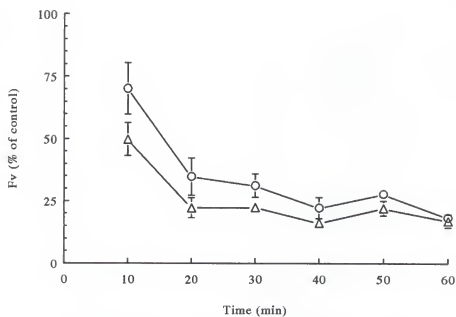


Figure 13. Effect of 20 μ M diquat on the Fv of paraquat-R (○) and -S (Δ) American black nightshade.

Table 7. Comparison of chlorophyll fluorescence *a* in leaf disks of the paraquat-S and -R biotypes incubated in 20 μ M diquat.

Fluorescence	Biotype		P > F
	Sensitive	Resistant	
	% control		
F _o	689.9	87.9	0.0620
F _p	53.7	57.8	0.0601
F _v	24.9	34.1	0.0028
F _s	86.2	86.5	0.8283

Table 8. Time-dependent effect of 1.7 mM paraquat on chlorophyll *a* fluorescence of leaf disks from a S and an R biotype.

Time min	Relative Chlorophyll Fluorescence					
	Sensitive			Resistant		
	F _o	F _p	F _s	F _o	F _p	F _s
0	64.8±4.7	119.8±4.0	82.8±5.6	67.8±4.6	138.5±3.8	83.7±3.6
10	62.7±3.4	94.3±3.6	75.0±4.8	64.2±3.6	119.5±5.8	79.5±3.4
20	64.2±3.7	84.7±5.0	75.2±4.7	65.2±5.2	103.0±5.4	81.2±5.1
30	65.5±3.0	80.8±2.5	73.2±3.2	65.2±4.6	102.7±3.4	78.7±3.9
40	65.2±4.1	80.3±3.7	75.7±3.7	65.0±2.5	102.8±3.9	79.7±2.7
50	61.7±3.7	76.0±3.3	73.3±3.4	62.0±3.7	90.3±3.1	74.7±4.1
60	63.0±4.0	80.0±3.9	73.7±3.6	64.7±3.3	96.3±4.1	77.0±4.3

(Fig. 14). However, this appeared to be due to inherently higher fluorescence by disks from untreated R plants (0 time). The possibility of a modification of the site of action cannot yet be discounted, but such a change is likely to result in a greater degree of resistance than was observed. Like diquat, there was no interaction between biotype and time, indicating that paraquat caused F_v to decrease at the same rate in both biotypes (Fig. 14). This indicated that paraquat as well as diquat are capable of rapid penetration to the site of action in both biotypes. In addition, these two experiments provided evidence of bipyridinium reduction at the site of action.

Concentration-dependent effects of diquat and paraquat

The concentration-dependent effects of diquat and paraquat were also investigated. Data from preliminary experiments are summarized in Tables 9 and 10,

respectively. Increasing the rates of diquat and paraquat in the bathing solution had no effect on F_o indicating that in these experiments both biotypes had similar levels of chlorophyll per unit leaf area. In both biotypes, F_p decreased as the herbicide rates increased, but the rate of decrease was not the same. F_p decrease was more rapid in the S biotype. F_s decreased with increasing concentrations of diquat and paraquat with no biotypic difference. Figures 15 and 16 illustrate the concentration-dependent effect of diquat and paraquat on F_v . Diquat and paraquat were more effective in suppressing F_v in the S biotype than in the R biotype. The concentration-dependent experiment was repeated 2 more times. A more convenient incubation period of 20 minutes was used and an additional concentration was added for the R biotype in order to determine whether fluorescence could be suppressed to the same degree as the S biotype. F_o , F_p , F_v and F_s were all higher in the R biotype than the S biotype (Table 11). F_o , F_p and F_s decreased in a similar manner as paraquat concentration was increased (Table 12). When F_v was expressed as a percentage of the control, there was an interaction ($P < 0.05$) between biotype and concentration (Fig. 17). Higher concentrations of paraquat were required by the R biotype to produce an equivalent decrease by the S biotype. Since the time-dependent effect of diquat and paraquat revealed that the rate of bipyridyl penetration to the site of action was the same, the effect of concentration can be interpreted to mean that the resistance mechanism is related to chloroplast photochemistry rather than exclusion or sequestration.

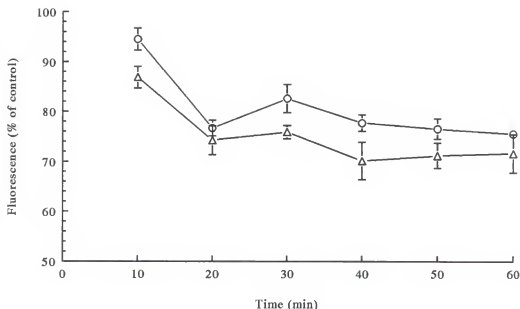


Figure 14. Effect of 1.7 mM paraquat on Fv of a R biotype (○) and a S biotype (Δ) of American black nightshade.

Table 9. Concentration-dependent effects of diquat on chlorophyll *a* fluorescence by leaf disks of the paraquat-S and -R biotypes dark-incubated in the diquat solution for 30 min.

Conc. (M)	Relative Chlorophyll Fluorescence					
	Sensitive			Resistant		
	F _o	F _p	F _s	F _o	F _p	F _s
0	63.0±2.4	127.7±6.3	86.0±3.3	62.5±2.2	135.8± 3.0	82.0±1.9
2E-8	58.5±1.0	119.5±2.3	79.0±0.9	58.7±2.2	136.0± 3.6	78.7±1.7
2E-7	60.0±1.5	129.0±5.0	81.7±2.4	57.5±1.6	127.0± 5.0	75.0±2.1
2E-6	59.2±3.2	97.5±5.4	76.0±2.6	59.0±2.3	129.3±10.3	81.0±5.0
2E-5	62.2±5.5	75.7±3.7	71.5±2.3	56.0±2.5	105.7± 8.8	71.2±3.8
2E-4	57.0±2.2	71.5±2.5	69.2±2.1	57.2±1.0	91.5± 2.2	71.7±0.8
2E-3	55.5±1.3	67.2±1.3	67.2±1.3	58.5±2.7	88.5± 2.7	70.7±2.8

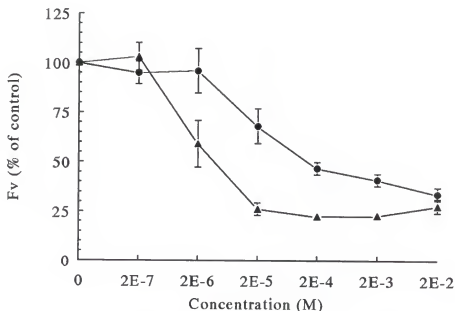


Figure 15. Concentration-dependent effects of diquat on F_v of leaf disks from paraquat-R (●) and -S (▲) biotypes of American black nightshade incubated in darkness for 30 min.

Table 10. Concentration-dependent effect of paraquat on chlorophyll a fluorescence when leaf disks were dark-incubated in the paraquat solution for 30 min.

Conc. (M)	Relative Chlorophyll Fluorescence					
	Sensitive			Resistant		
	F_o	F_p	F_s	F_o	F_p	F_s
0	63.0±1.1	127.5±4.8	85.2±1.8	59.7±1.9	123.0± 5.6	76.5±3.1
2E-8	59.5±1.8	98.7±6.5	78.2±3.2	60.7±1.7	121.5± 6.8	79.0±2.0
2E-7	56.7±1.9	95.0±1.0	76.2±1.7	54.2±3.4	105.5±13.1	72.2±5.0
2E-6	57.5±3.6	86.5±6.6	74.7±3.1	60.2±1.6	122.2± 7.5	79.5±3.4
2E-5	57.5±3.3	77.2±2.7	71.5±2.9	55.5±3.0	103.2±11.0	71.2±3.9
2E-4	57.2±0.9	74.5±1.8	70.5±1.0	58.2±2.9	110.0± 3.3	76.5±2.7
2E-3	58.7±1.7	76.7±3.4	71.7±1.8	60.0±3.6	97.7±12.1	76.0±5.5

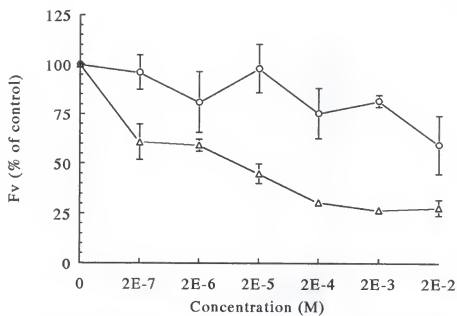


Figure 16. Concentration-dependent effects of paraquat on Fv of leaf disks of a R (○) and an S (Δ) biotype incubated in darkness for 30 min.

Table 11. Effect of paraquat on chlorophyll *a* fluorescence in leaf disks of a S and R biotype of American black nightshade.

Relative Fluorescence	Biotype		P > F
	Sensitive	Resistant	
F _o	60.7	63.2	0.0001
F _p	110.5	133.2	0.0001
F _v	49.8	70.0	0.0001
F _s	75.6	81.7	0.0001

Table 12. Concentration-dependent effect of paraquat on chlorophyll *a* fluorescence when leaf disks were incubated in darkness over a 20 min period.

Conc. (M)	Relative Chlorophyll Fluorescence					
	Sensitive			Resistant		
	F ₀	F _p	F _s	F ₀	F _p	F _s
0	64.5±1.6	131.6±5.2	82.0±2.1	65.9±1.8	152.5±2.2	88.4±4.5
2E-8	61.7±1.7	121.1±6.0	78.1±2.1	64.4±1.2	145.6±2.7	82.4±1.5
2E-7	60.6±1.4	126.0±3.5	78.6±1.5	62.7±1.7	145.6±2.8	83.4±2.8
2E-6	59.1±2.2	113.5±4.6	76.5±3.1	64.2±2.5	141.4±3.0	82.9±2.5
2E-5	61.4±1.0	101.7±1.8	73.1±2.2	63.0±1.4	135.8±4.5	81.9±1.5
2E-4	60.4±1.1	91.5±2.3	71.0±2.0	64.6±1.8	127.1±3.6	81.0±1.8
2E-3	57.0±1.4	88.1±2.3	70.0±2.5	61.0±1.8	117.2±4.1	78.9±2.3
2E-2	-	-	-	59.7±1.3	100.6±3.0	74.5±2.8

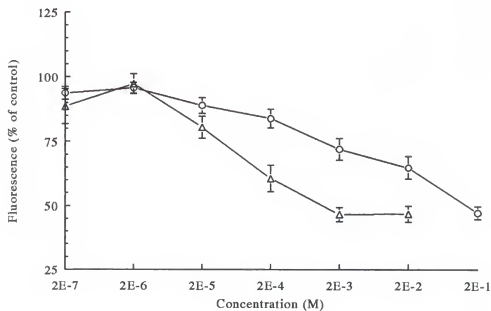


Figure 17. Concentration-dependent effects of paraquat on Fv of leaf disks from R (○) and S (Δ) biotypes incubated in darkness for 20 min.

Discussion

The measurement of ethane production was used as an indirect assay for paraquat penetration to and activity at the site of action. The stimulation of lipid peroxidation in both biotypes indicated that paraquat penetrated to the site of action and was capable of inducing phytotoxicity in the R biotype in a similar manner to the S biotype. Ethane evolution was also used to compare susceptibility to lipid peroxidation in S and R *C. canadensis* (Lehoczki et al., 1992). In accordance with our results, they found that in the absence of paraquat there was no difference in ethane production between biotypes. With the addition of paraquat there was an 8-fold increase in ethane production by the S biotype of *C. canadensis* compared with only a 3-fold increase in the R biotype. Similar results were reported by Pölös et al. (1988) who found increased ethane and malondialdehyde (MDA) production in the paraquat-S biotype of *C. canadensis* when leaves were treated with 100 μ M paraquat. The paraquat-R biotype, which is also resistant to atrazine, was unaffected. Similarly, no ethane was produced by paraquat-R *C. bonariensis* (Youngman and Dodge, 1981).

Electrolyte leakage, MDA formation and chlorophyll bleaching have also been used to compare paraquat activity in R and S *C. bonariensis* (Vaughn and Fuerst, 1985). While there was only a small change in these components by the R biotype over control levels when leaf disks were treated with 10 μ M, considerably more damage occurred in S biotype. Paraquat activity has also been examined in isolated chloroplasts of *C. bonariensis* (Jansen et al., 1990; Shaaltiel and Gressel, 1987b).

Untreated chloroplasts of the R biotype evolved less ethane than the S biotype. The addition of paraquat caused a greater increase in ethane in the S biotype. The use of isolated chloroplasts eliminated the possibility of cytoplasmic sequestration. The limited lipid peroxidation of the R biotype was coincident with constitutively elevated protective enzymes, which were thought to be the primary mechanism of resistance (Jansen et al., 1990; Shaaltiel and Gressel, 1987b). Another group working with this species has been unable to corroborate these findings (Norman et al., 1993). Elevation of constitutive levels of protective enzymes is not a plausible explanation of paraquat resistance in American black nightshade since in our assays these enzymes were inhibited by KCN.

Norman et al. (1993) indirectly investigated the ability of paraquat to interact with the site of action by measuring MDA production, and directly by EPR analysis and polarographically (partial PSI assay of oxygen consumption) in isolated thylakoids. They found no evidence of a change in the site of action. This supported evidence derived from a PSI partial reaction with isolated chloroplasts that there was no change at the site of action (Fuerst et al., 1985). This technique has also been used to assess paraquat activity in *H. glaucum* (Powles and Cornic, 1987). No evidence has as yet been provided that implicates mutation of the site of action in conferring paraquat resistance.

The rapid phytotoxic activity of paraquat requires the photolysis of water (Homer et al., 1960; Mees, 1960). Light is the energy source that drives the release of electrons from water molecules, providing the reducing power to generate the

radical form of the herbicide. The bipyridinium herbicides are capable of killing non-photosynthetic tissue as well as dark-incubated green tissue (Summers, 1980). The process is much slower and respiration is thought to be the source of the reducing power (Homer et al., 1960; Mees, 1960). The electrolyte leakage data from American black nightshade demonstrated that the difference in paraquat activity between biotypes in the dark was small. This indicated that a sufficient quantity of paraquat must have remained unsequestered in both biotypes, resulting in the observed membrane damage in the dark. The biotypic difference in paraquat activity under illumination was considerably greater. This suggested that the resistance mechanism was light-dependent and not sequestration or metabolism.

Photosynthetic electron transport can be assessed with great sensitivity in a nondestructive manner by measuring chlorophyll fluorescence (Smillie and Gibbons, 1981). F_0 depends to a large degree on the chlorophyll content of the leaf per unit leaf area (Lichtenthaler, 1988). F_v is defined as the increase in fluorescence above the F_0 to F_p . Its variable nature arises from its responsiveness to changes in electron flow through PSII (Smillie and Gibbons, 1981). Thus, the determination of F_v is a useful technique for measuring the effect of various stresses on photosynthetic electron transport. Conditions that inhibit electron flow on the photoreducing side of PSII increase F_v , while those that inhibit electron flow on the photooxidizing side reduce F_v (Smillie and Gibbons, 1981). The measurement of F_v has been used as a direct assay of the presence of paraquat at the site of action in PSI (Munday and

Govindjee, 1969) and is a useful tool in investigations of the mechanism of paraquat resistance.

The analysis of chlorophyll *a* fluorescence in leaf disks of American black nightshade confirmed that paraquat does penetrate to and is reduced at the site of action of the R biotype. However, both diquat and paraquat were more effective in suppressing F_v in the S biotype. Chlorophyll fluorescence has also been used to assess paraquat activity in paraquat-R *C. bonariensis* and *C. canadensis*. Although these results were comparable to those with American black nightshade, the interpretations were not always in accord. In *C. bonariensis*, fluorescence quenching was observed in both biotypes with much higher concentrations of paraquat required to effect the response in the R biotype (Fuerst et al., 1985; Vaughn et al., 1989). The inability of the R biotype to quench fluorescence to the same degree as the S biotype was regarded as direct evidence that the herbicide did not reach the site of action.

Decrease in F_v of the R biotype of *C. canadensis* occurred within 7 minutes (Lehoczki et al., 1992). Even though more fluorescence suppression occurred in the S biotype, the workers felt that suppression in the R biotype was a definite indication of paraquat penetration of the chloroplasts of the R plants and interaction with the site of action. This was consistent with our interpretation of similar data in American black nightshade. However, they concluded that the data supported the protective enzyme mechanism in combination with a mechanism for the elimination of paraquat activity (Lehoczki et al., 1992). Isolated chloroplasts from an S biotype and an R

biotype of *C. canadensis* co-resistant to paraquat and atrazine quenched maximum fluorescence (F_m) to the same extent (Pölös et al., 1988). This also was true for short-term measurement on intact leaves. However, when measured at 24 h after paraquat treatment, less quenching was apparent in the R biotype. The quenching of chlorophyll fluorescence in the R biotype was a transient effect, but was not reversible in the S biotype (Lehoczki and Szigeti, 1988; Lehoczki et al., 1992; Pölös et al., 1988; Szigeti et al., 1988). The gradual recovery of chlorophyll fluorescence levels was thought to indicate the progressive elimination of paraquat activity.

There is an inverse relationship between fluorescence and the rate of photosynthesis. When photosynthesis is optimized, most of the absorbed light energy is utilized and there is very little fluorescence (Lichtenthaler, 1988). It is thus conceivable that the higher F_p and F_v values obtained for the R biotype of American black nightshade may be due to differences in photosynthetic electron transport. If photosynthetic electron transfer is impaired in paraquat-R American black nightshade, the higher levels of F_v imply that the causal mutation is located between PSII and PSI. Thus it is likely that a PSI partial reaction will not detect it. Midmore and Prange (1992) have found that plants grown under conditions of low temperature and low irradiance had low rates of photosynthesis and correspondingly higher F_p than similar plants grown at higher temperature. The increased F_p and F_v was felt to be due to less efficient movement of electrons through PSII. Evidence of reduced rate of electron transfer between Q_A and Q_B and of a 20% reduction in the relative rate of electron transport between water and plastoquinone has been reported for the R

biotype of *C. canadensis* (Lehoczki et al., 1992). However, it was apparently not considered important enough to warrant mention in the conclusions. There was no difference in the rate of electron transfer between Q_A and Q_B when an S and an R biotype of *C. canadensis* with atrazine co-resistance were compared (Pölös et al., 1988).

Based on the data collected from the measurement of ethane, electrolyte leakage and fluorescence in the presence of paraquat it can be concluded that paraquat does enter the chloroplast of the R biotype. The involvement of constitutive protective enzymes was eliminated. Paraquat was capable of diverting electrons from PSI and exhibited phytotoxic action, albeit at a lower level than the S biotype. The results suggest that the resistance mechanism is chloroplastic and may be related to differences in electron transport or a change at the site of action. However, cytoplasmic mechanisms such as vacuolar sequestration or paraquat metabolism that may reduce the amount of paraquat that penetrates to the site of action could not be ruled out unequivocally.

CHAPTER 4

DIFFERENTIAL PHOTOSYNTHETIC ELECTRON TRANSPORT AND OXIDATIVE STRESS IN RESISTANT AND SENSITIVE BIOTYPES OF AMERICAN BLACK NIGHTSHADE

Introduction

Phytotoxicity of bipyridiniums is caused by the formation of toxic oxygen radicals resulting from the autooxidation of the bipyridyl radical. Superoxide (O_2^-) is the first product of the reaction between the bipyridyl radical and oxygen. In vitro production of O_2^- from the autooxidation of diquat and paraquat radicals has been demonstrated (Giannopolitis and Ries, 1977; Stancliffe and Pirie, 1971). The involvement of O_2^- in mediating paraquat toxicity was proposed because it was determined that the radical was sufficiently long-lived to promote the observed phytotoxic effect (Farrington et al., 1973). Several bipyridyl compounds were shown to stimulate the reduction of ferricytochrome *c* by isolated thylakoids, a reaction that was inhibited by superoxide dismutase (Asada et al., 1974). Youngman et al. (1979) also provided in vivo evidence that O_2^- was involved in promoting paraquat toxicity.

Superoxide can participate directly in oxidation and reduction reactions with cell components leading to toxic effects. Superoxide also reacts with H_2O_2 through the iron-catalyzed Haber-Weiss reaction giving rise to the more reactive hydroxyl radical ($OH^\bullet$). The $OH^\bullet$ reacts indiscriminately with almost all cell components and

initiates and propagates the peroxidation of cell membranes. Hydroxyl radicals have been detected in paraquat-treated plants (Babbs et al., 1989). The dismutation of O_2^- spontaneously or by catalytic action of SOD results in the formation of H_2O_2 . A strong oxidant, H_2O_2 inhibits CO_2 fixation through oxidation of sulfhydryl groups of the enzymes that catalyze the process (Kaiser, 1979). The formation of O_2^- is an unavoidable consequence of photosynthetic electron transport due to the leakage of a small amount of electrons to O_2 in a process known as the Mehler reaction (Asada and Kiso, 1973). The resultant active oxygen species are scavenged by SOD followed by the enzymes of the Halliwell-Asada pathway (Halliwell, 1987). The enzymes of this pathway are ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase. Paraquat stimulates radical production to levels that overwhelm the detoxification process.

The results presented in chapter three strongly implicated the involvement of chloroplast photochemistry in American black nightshade paraquat resistance. In light of evidence suggesting that electron transport was reduced in the R biotype, the possibility existed that the paraquat-R and -S biotypes of American black nightshade differed in their ability to generate toxic oxygen species. Therefore, the objectives of these experiments were two-fold: (1) to confirm whether there were inherent differences between the rates of photosynthetic electron transport of the paraquat-R and the -S biotypes, and (2) to determine whether the biotypes differed in their ability to generate oxygen stress.

Materials and Methods

Chloroplast and Thylakoid Membrane Isolation

Each group of R and S plants used in these experiments were scored for resistance or sensitivity to paraquat and held in darkness for 24–48 h in order to reduce the size of starch granules in the chloroplasts. Different individuals were used for each assay. Two or three of the most recently expanded leaves per plant were detached, washed and blotted dry. Leaves were sliced thinly and placed in 150 mL of partially frozen grinding (GR) buffer. The grinding buffer consisted of 0.4 M sorbitol, 50 mM MOPS-KOH pH 7.5, 10 mM $MgCl_2$ and 1% bovine serum albumin (BSA). This grinding buffer was chosen because it was used successfully in the isolation of chloroplasts from a related species, *S. nigrum* (Siegenthaler and Mayor, 1992). Leaf material was homogenized with a Waring blender in two 5 s bursts. The homogenate was filtered through 4 layers of cheesecloth and 2 layers of nylon screen moistened with GR buffer. Filtrate was poured into chilled 50 mL polypropylene centrifuge tubes and centrifuged at 2800 rpm using an IEC clinical centrifuge with a swing-out head rotor (International Equipment Company, Needham, MA). All centrifugation, unless otherwise indicated, was done at 2800 rpm with the IEC clinical centrifuge. Centrifuge tubes filled with ice were placed in the rotor cups between centrifugations so that all operations were performed at low temperature. The filtrate was centrifuged for 3 min. The supernatant was discarded and the pellet resuspended in GR buffer. For the determination of O_2^- formation it was necessary to reduce the

possibility of mitochondrial contamination of the chloroplast preparation because mitochondrial cytochrome *c* oxidase reverses the reaction of interest (Asada, 1984). Therefore, a Percoll gradient was prepared by combining 50% 2X GR buffer with 50% Percoll (Sigma Chemical Company, St Louis, Mo). The gradient was generated by centrifugation of 25 mL of the Percoll mixture at 20,000 rpm for 40 min, using a Beckman model J2-21 refrigerated centrifuge (Beckman Instruments Inc., Fullerton, CA). The resuspended chloroplasts were gently layered onto the gradient. Centrifugation for 15 min in the Beckman centrifuge at 4°C with a fixed angle JA 20 rotor did not produce very distinct layers, and so the IEC clinical centrifuge with its swing-out head rotor was employed in the second experiment. The top layers were aspirated off and the lower green band retrieved with a wide bore Pasteur pipet. This was diluted to 50 mL with chloroplast wash buffer and centrifuged for 3 min. The wash buffer consisted of GR buffer minus the BSA. The pellet was resuspended and washed again. After resuspension in 5 mL chloroplast wash buffer, the chloroplasts were examined by means of phase contrast microscopy to confirm integrity of the organelles.

Chloroplasts were lysed by incubating them for 2 min in 5 mL chloroplast wash buffer plus 45 mL 20 mM potassium phosphate buffer, pH 7.8. Thylakoids were collected by centrifugation for 5 min. Thylakoids were resuspended in a 20 mM potassium phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraacetic acid (EDTA). This buffer was used to deplete the thylakoids of stromal SOD. After 1 h at 4°C, the thylakoids were collected by centrifugation for 5 min and the SOD

removal step repeated with fresh buffer. On completion of the second wash step the thylakoids were collected by centrifugation as previously described and resuspended in a small quantity of 50 mM potassium phosphate buffer, pH 7.8, containing 10 mM NaCl.

For the electron transport experiments, instead of a continuous gradient, a Percoll cushion was used to purify the chloroplasts. It consisted of 50% GR buffer and 40% Percoll. The resuspended chloroplasts were layered onto the Percoll cushion and centrifuged for 3 min. The pellet was resuspended in 5 mL chloroplast wash, which was then diluted to 50 mL with cold distilled water. Chloroplasts were allowed to lyse over the next 5 min, then the thylakoids were collected by centrifugation. They were resuspended in 1 mL of chloroplast wash buffer. Chlorophyll was determined after Bruinsma (1961).

Electron Transport

Ferricyanide reduction

The photoreduction of ferricyanide to ferrocyanide was one of two methods used to compare photosynthetic electron transport in the 2 biotypes. The photoreduction was conducted as per Siegenthaler and Mayor (1992) and ferrocyanide was detected after Avron and Shavit (1963). The 1 mL reaction mixture consisted of 0.3 M sorbitol, 50 mM MOPS-KOH pH 7.2, 5 mM $MgCl_2$, 5 mM NH_4Cl and 0.5 mM $K_3Fe(CN)_6$. Because of differences in yield of chloroplasts, 20 μg chlorophyll mL^{-1} were used in experiment 1 and 10 μg chlorophyll mL^{-1} were used in the second

experiment. Thylakoids were illuminated at $350 \mu\text{E m}^{-2} \text{s}^{-1}$ with a 300 W incandescent lamp. A 3.5 cm deep water filter was used to reduce heating by IR radiation and red light was provided by means of a red filter that eliminated wavelengths below 650 nm. At intervals of 0.5, 1, 1.5, 2 and 2.5 min, the reaction was stopped by the addition of 100 μL of 30% trichloroacetic acid (TCA). Each treatment was replicated 3 times. An appropriate control was prepared for each biotype by addition of the TCA without illumination.

Accumulation of ferrocyanide was monitored spectrophotometrically. The reaction mixture was transferred to micro-centrifuge tubes and a clear supernatant obtained by centrifugation at 12000 rpm for 1 min, using a Fisher micro-centrifuge model 59A (Fisher Scientific, Pittsburgh, PA). An aliquot of the supernatant was sampled: 300 μL in the first experiment, 600 μL in the second. To the aliquot, 1.8 mL of distilled water were added, followed by 0.9 mL of reagent mixture. The stock reagents were 3 M sodium acetate (pH 6.2), 0.2 M citric acid, 3.3 mM FeCl_3 dissolved in 0.1 M acetic acid and 4,7-diphenyl-1,10-phenantroline, sulfonated. The concentration of the last reagent was 100 mg per 30 mL water. The reagents were mixed in the ratio 2:2:1:1 just prior to use. After addition of the reagent mixture, the solutions were exposed to ambient room light for 5 minutes for color development, then they were covered with foil until absorbance was determined. This was done at 535 nm using a Shimadzu UV-Visible recording spectrophotometer, model UV 160 (Shimadzu Corporation Spectrophotometric Instruments Plant, Kyoto, Japan).

The data were expressed as $\mu\text{mol ferrocyanide mg}^{-1}$ chlorophyll using a molar absorption coefficient of 20,500 (Avron and Shavit, 1963). The data were analyzed as a randomized complete block design. ANOVA was performed and the means were regressed against time.

NADP⁺ reduction

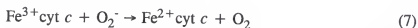
NADP⁺ reduction was successfully accomplished in only 2 of 4 studies. The procedure employed in the two successful studies was as follows. The first experiment was performed in 1 mL of reaction mixture containing: 40 mM tricine-NaOH (pH 7.8), 5 mM MgCl₂, 1 mM ADP, 10 mM Na₂HPO₄, 0.2 mM NADP⁺, 50 μg ferredoxin (Fluka Chemical, Ronkonkoma, NY) and thylakoids at 15 $\mu\text{g mL}^{-1}$. In addition to an untreated control, thylakoids were also exposed to 100 μM paraquat. Illumination was provided by a projector equipped with a 300 W lamp. Light was filtered through 6 cm of water (flat glass bottle containing deionized water) to reduce heating due to IR radiation and a red filter was used to exclude wavelengths of less than 650 nm. The light intensity was 250 $\mu\text{E m}^{-2} \text{s}^{-1}$. The reaction mixture was exposed to light for 2.5, 5.0, 7.5, 10, 15 and 20 min. NADPH formation was detected spectrophotometrically by absorbance increase at 340 nm. In order to compensate for the gradual decrease in the absorbance of the blank over the course of the assay, three replicate blanks were prepared with and without paraquat for each biotype. The absorbance of these were determined without illumination and used to adjust sample absorbances. Data were expressed as $\mu\text{mol NADPH mg}^{-1}$ chlorophyll

using a molar absorptivity coefficient of 6,200. Each treatment was replicated 3 times and the experiment was analyzed as a randomized complete block design.

For the second experiment, several changes were made in the procedure. The chloroplasts were isolated on the day prior to use in the assay and so were more than 24 h old when used. The chlorophyll concentration was $5 \mu\text{g mL}^{-1}$ in a 3 mL reaction volume. The reaction volume was changed in order to make use of glass cuvettes that gave more precise readings than the disposable cuvettes used previously. Complete blanks were prepared with and without paraquat. Atrazine, a photosynthesis inhibitor was included at $50 \mu\text{M}$ in an attempt to stabilize the blank. Three replicate cuvettes were exposed to light and repeated absorbance determinations were done at 3, 6, 9 and 12 min. As a result, a more stringent analysis of the data was required. A repeated measures analysis was done using a split-plot design. One replicate with thylakoids from the R biotype consistently gave a negative absorbance that did not change over time. The data for this replicate were not included in the analysis. The means for the nontreated R biotype were based on two replicates.

Measurement of Superoxide Radicals

The procedure for measuring O_2^- generation was taken from Asada (1984). The assay involved the spectrophotometric determination, at 550 nm, of ferricytochrome *c* reduction to ferrocycytochrome *c* by O_2^- .



The experiment was conducted in a 2 mL reaction mixture, which consisted of 50 mM potassium phosphate, pH 7.8, 10 mM NaCl, 20 μ M ferricytochrome *c* and 100 μ M paraquat. An untreated control was included for comparison. For the first experiment, the chlorophyll content was 5 μ g mL⁻¹ and 10 μ g mL⁻¹ for the second experiment. The reaction mixture was illuminated as described previously for the NADP⁺ reduction. The illumination periods were 1, 2.5, 5, 7.5 and 10 min for experiment 1 and the small yield of chloroplasts restricted the number of replicates to two. In experiment 2, some additional illumination periods were used: 15 and 20 for the S biotype and 15, 20 and 40 min for the R biotype. The treatments were replicated 3 times in experiment 2.

The difference absorption coefficient between ferri- and ferrocytochrome *c*, used for expressing the data in terms of nmol, was 19,000 M⁻¹ cm⁻¹ (Asada, 1984). A complete blank plus 50 μ g mL⁻¹ SOD was prepared for each biotype. There was a gradual decrease in the absorbance of the blank resulting in a total decrease in absorbance of 0.049 for the S biotype and 0.031 for the R biotype over the course of the experiment. In the second experiment an attempt was made to adjust for the changes that were occurring in the blank. For the S biotype, the reading of the blank was noted when the sample absorbance was recorded so that it was possible to adjust for the changes from the original reading of the blank. For the R biotype the absorbance of the blank against water was read. This absorbance was used to adjust the sample readings which were also blanked against water. The data from the first experiment were analyzed as a randomized complete block, blocking on replicate.

The ANOVA procedure of SAS was used and standard errors were calculated. For the second experiment the GLM procedure was used for analyzing the raw data and the REG and NLIN procedures were used to regress the means.

Results

Electron Transport

Photosynthetic electron transport was compared using an artificial electron acceptor, ferricyanide, and the natural electron acceptor, NADP⁺. The reduction of ferricyanide occurred more rapidly in the S biotype than the R biotype (Fig. 18), indicating that the rate of electron transfer was faster in the S biotype.

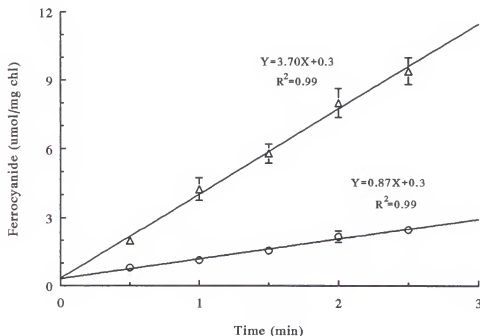


Figure 18. Reduction of ferricyanide to ferrocyanide in isolated thylakoids of paraquat-S (Δ) and -R (○) biotypes of American black nightshade.

Corroboration of these results was provided by the data from the experiments measuring NADP^+ reduction. Again, more rapid formation of NADPH by the S biotype provided evidence that electron transport was slower in the R biotype (Fig. 19). While the addition of paraquat reduced NADPH production in the S biotype, no effect was observed in the R biotype in the first experiment (Fig. 19). In the second experiment, there was a significant ($P < 0.05$) biotype by time interaction. Further analysis indicated that the effect of time was not significant in the R biotype, whereas NADPH accumulated with time in the S biotype (Fig. 20). The inclusion of $100 \mu\text{M}$ paraquat completely eliminated NADP^+ reduction in both biotypes (data not shown).

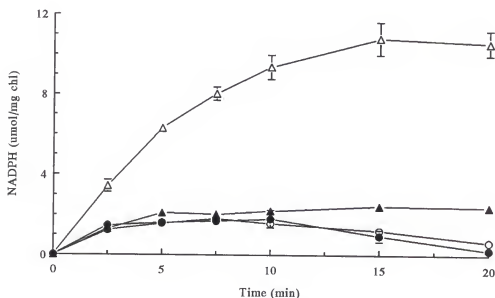


Figure 19. Production of NADPH in isolated thylakoids of paraquat-S (Δ) and -R ($\circ$) biotypes as measured by absorbance at 340 nm. Closed symbols indicate NADPH production in the presence of at $100 \mu\text{M}$ paraquat.

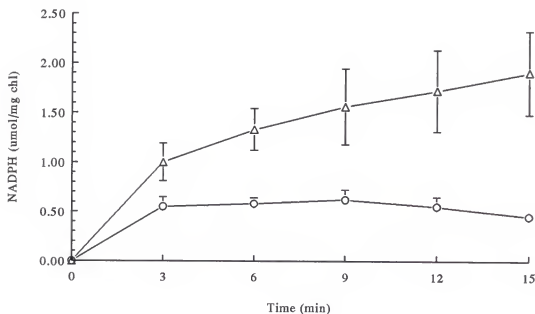


Figure 20. Production of NADPH in isolated thylakoids of paraquat-S (Δ) and -R (○) as measured by absorbance at 340.

The magnitude of the response in these two experiments varied because the chloroplasts used in the second experiment were slightly aged because they had been isolated the previous day. This tends to reduce the activity of the chloroplasts. Since the chloroplasts were isolated from two different groups of plants, this may also account for some of the observed variability in the response.

Oxygen Radical Generation

Similar results were obtained in both experiments in which O_2^- generation was measured. The data were not combined since the experiments were not performed over the same time period, and there were differences in the way in which the experiments were conducted. Figure 21 illustrates the data collected from the first

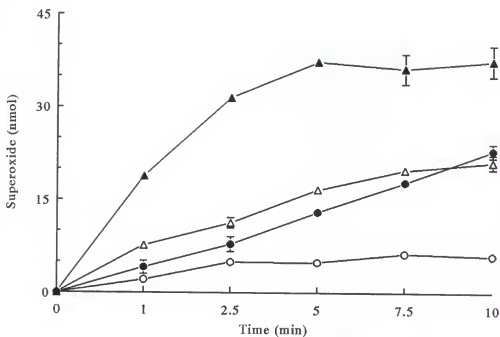


Figure 21. Comparison of superoxide production in the S (Δ) and R ($\circ$) biotypes. Closed symbols indicate paraquat treatment at 100 μ M. (Experiment 1).

assay. Superoxide was generated by thylakoids of both biotypes in the absence of paraquat. The interaction between biotype and time ($P < 0.05$) showed that the rate of radical production in the S biotype exceeded that of the R biotype. The addition of paraquat elevated O_2^- production in both biotypes with greater stimulation occurring in the S biotype (Fig. 21). The trends of the second experiment were similar to those of the first experiment. In the absence of paraquat the S biotype had a high rate of O_2^- production that was enhanced by the addition of paraquat (Fig. 22a). The R biotype had a lower rate of radical formation than the S biotype when no paraquat was present. Addition of paraquat stimulated the rate of O_2^- generation (Fig. 22b). It was clear from both Figures 21 and 23 that in the presence of 100 μ M paraquat,

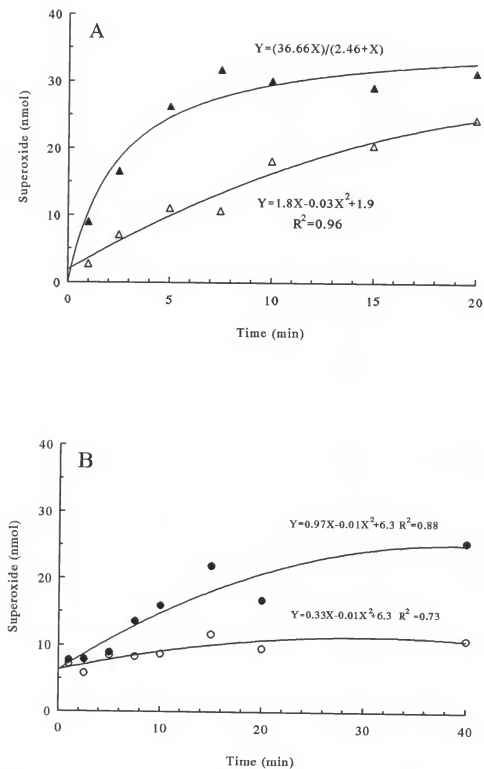


Figure 22. Comparison of superoxide production in: [A] the S biotype with 100 μ M paraquat ($\blacktriangle$) or without ($\triangle$), and [B] the R biotype with 100 μ M paraquat ($\bullet$) or without ($\circ$). (Experiment 2).

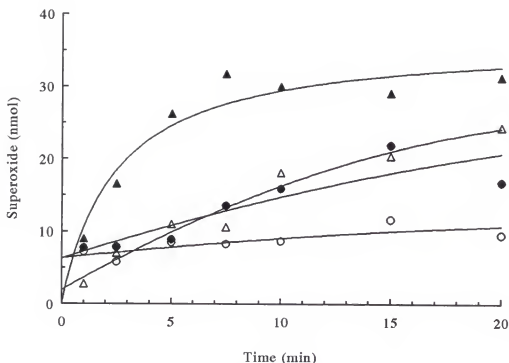


Figure 23. Comparison of superoxide production in the S (Δ) and R (○) biotypes. Closed symbols indicate paraquat treatment at 100 μ M. (Experiment 2).

isolated thylakoids from the R biotype generated levels of O_2^- similar to those of the S biotype in the absence of paraquat. This suggests that the addition of paraquat to the R biotype produces a level of oxygen stress that can be withstood by the plant without irreparable damage occurring. The similarity in response when electron transport and radical generation were measured seem to indicate that the restricted oxygen stress in the R biotype is due to reduced electron transport.

Discussion

The results presented herein provide evidence of a slower rate of electron transport in the paraquat-R biotype of *S. americanum*. This impairment results not only in a

lower rate of the Mehler reaction but also in reduced oxygen stress in the presence of paraquat when measured as O_2^- . The data of Yanase et al. (1992) support the argument that reduced photosynthesis translates into reduced paraquat toxicity. They showed, by measuring electrolyte leakage, that paraquat toxicity can be alleviated by photosynthesis inhibitors such as DCMU. Toxicity is reduced because the inhibitors reduce the electron supply from the electron transport system to paraquat.

In the absence of a direct measurement of the activity of the protective enzymes (SOD and the Halliwell-Asada enzymes) in the R biotype of *S. americanum*, it is assumed that either the constitutive levels are adequate to cope with the oxidative stress or that constitutive levels provide sufficient protection to maintain cell integrity until existing levels are augmented. The latter is plausible since 50 μ M paraquat has been shown to cause enhanced levels of SOD mRNAs in illuminated *Nicotiana plumbaginifolia* (Tsang et al., 1991). The increase was 40-fold in the chloroplasts, 30-fold in the mitochondria, and 15-fold in the cytoplasm. Unpublished data from this laboratory (T.A. Bewick and K.L. Smith) also supports this possibility. They found that constitutive levels of the antioxidant ascorbate were the same in both biotypes of *S. americanum*. However, while application of paraquat caused a net loss of ascorbate in the S biotype, ascorbate depletion was transient in the R biotype. Subsequently, ascorbate was observed to accumulate in excess of the constitutive level (Bewick and Smith, unpublished).

Paraquat resistance due to impaired electron transport as observed here in *S. americanum* is unique. Lower rates of electron transport have been reported in leaf

disks and protoplasts of a paraquat-R biotype of *C. canadensis*. However, there were no differences at the chloroplast level and the mechanism of resistance was attributed to enhanced constitutive levels of protective enzymes (Itoh and Matsunaka, 1990). Photosynthesis has also been assessed in other paraquat-R biotypes. Szigei and Lehoczki (1992) compared photosynthetic electron transport between PSII and PSI in two R biotypes and one S biotype of *C. canadensis* by evaluation of P700 (reaction center of PSI) absorbance changes. They found no evidence to support the occurrence of a mutation along the electron transport chain from plastoquinone to the cytochrome b_6f . In another report on *C. canadensis*, CO_2 fixation rates at time zero in the R and S biotypes for the same species as read from a figure were 1.75 and $2.25 \text{ nmol cm}^{-2} \text{ s}^{-1}$, respectively. However, there was no indication of whether the rates were significantly different (Pölös et al, 1988). In general, the focus of investigations of photosynthetic electron transport and CO_2 fixation has been to determine if target site mutation has occurred or to compare the extent to which paraquat inhibited CO_2 fixation in an R and an S biotype. Such experiments with *C. bonariensis*, *H. glaucum*, *H. leporinum* yielded no evidence of differences in electron transport that could be implicated in the resistance phenomenon (Fuerst et al., 1985; Norman et al., 1993; Powles and Cornic, 1987; Preston et al., 1992).

In summary, a unique mechanism is responsible for paraquat resistance in *S. americanum*. Photosynthetic electron transport is impeded in the R biotype. This causes a level of oxidative stress, in the presence of $100 \mu\text{M}$ paraquat, similar to that of the S biotype in the absence of paraquat. This magnitude of stress is probably well

within the protective capacity of the enzyme systems of R biotype and so phytotoxicity is not observed until sufficient paraquat is applied, to raise the oxidative stress to a level with which the plant cannot cope.

As long as paraquat remains in the chloroplast, oxygen toxicity will be promoted. As a result, a continuous allocation of NADPH would be required to drive the scavenging of toxic oxygen species by the Halliwell-Asada enzymes. It may be that sublethal doses of paraquat improve the efficiency of electron transport in the R biotype so that both NADPH and paraquat radicals are formed. However, lethal doses of paraquat would be expected to totally eliminate NADPH formation so that all scavenging of toxic oxygen ceases. Alternatively, the gradual elimination of paraquat from the chloroplast may occur by vacuolar sequestration.

CHAPTER 5 SUMMARY AND CONCLUSIONS

Reports by Collier County tomato farmers of reduced control of *S. americanum* by paraquat (Gilreath and Stall, 1987) encouraged efforts to determine the cause of the problem. The possibility of an interaction with copper-containing pesticides was investigated (Bewick et al., 1990a,b). While some of the pesticides did reduce the efficacy of paraquat, in the absence of copper pesticides the progeny of plants collected from Collier County were 12-fold more resistant to paraquat than plants from an unselected Gainesville population (Bewick et al., 1990a). This suggested that as for other paraquat-resistant weeds, the high selection pressure of multiple sprays of paraquat per season had permitted the establishment of a resistant population in Collier county.

The purpose of this study was to determine the mechanism of paraquat resistance in *S. americanum*. A number of mechanisms have been previously proposed to explain paraquat resistance in other weed species. Therefore, the hypothesis tested in this study was: paraquat resistance in *S. americanum* can be explained by a previously established mechanism. There were two main objectives: (1) to determine whether paraquat could penetrate to the site of action and undergo normal reduction in the R biotype, and (2) to compare the ability of the R biotype and the S biotype to generate and defend against oxidative stress.

Mechanisms that would restrict the penetration of paraquat into the cytoplasm include: (1) reduced cuticular penetration, (2) apoplastic sequestration, and (3) exclusion by the plasmalemma. Experiments were conducted to determine whether resistance was due to reduced cuticular absorption. The cuticle did not appear to be a barrier to paraquat uptake by leaf disks. The biotypes did not differ in the amount of paraquat that was absorbed, and paraquat uptake in *S. americanum* was characterized by a quadratic relationship. Examination of efflux kinetics showed that efflux from both biotypes occurred at a rapid rate within the first 10 min, then tapered off. However, the rate of paraquat efflux was significantly greater in the S biotype. This pattern of efflux could arise from either greater apoplastic sequestration or greater absorption of paraquat into the cytoplasm by the R biotype. To distinguish between these two possibilities a study of paraquat compartmentalization was conducted. There was more unbound paraquat in the apoplast of the S biotype. More paraquat was adsorbed in the apoplast of the R biotype than the S biotype. However, it is unlikely that rapid sequestration of paraquat by apoplastic adsorption accounts for resistance in *S. americanum* because the level of paraquat in the cytoplasm was similar in both biotypes. In the R biotype, this was 3-fold more paraquat than was bound in the apoplast. The biotypes had a similar level of cytoplasmic paraquat. Thus, the data did not appear to support either apoplastic sequestration or exclusion by a less permeable plasmalemma as a possible primary mechanism of resistance.

In order to determine whether paraquat could penetrate to the site of action in the R biotype and was capable of undergoing reduction, a number of biochemical

responses to paraquat treatment were compared in the two biotypes. Ethane evolution was measured as an index of lipid peroxidation. In the absence of paraquat there was no difference in ethane production by the two biotypes and ethane was produced at the same rate by both biotypes. The addition of paraquat caused increased ethane production in both biotypes. The rate of ethane production was greater in the S biotype. The incremental increase in ethane that occurred on raising the paraquat concentration from 8.5 μM to 85 μM was smaller than the increase measured on raising the concentration from 0 to 8.5 μM . Since the protective enzymes, superoxide dismutase and ascorbate peroxidase, were inhibited by inclusion of KCN in the incubation solution, it is unlikely that the differential response observed was due to enhanced radical scavenging.

In darkness, time-dependent electrolyte leakage from paraquat-treated leaf disks was the same for the first 12 h. After 12 h, electrolyte leakage increased at a more rapid rate in the S biotype than the R biotype. Illuminated paraquat-treated leaf disks of the S biotype leaked ions faster than those of the R biotypes, so that 100% leakage occurred within 12 h for the S biotype and by 24 h in the R biotype. Concentration-dependent ion leakage was also investigated in darkness and in light. Whereas the differences in ion leakage between biotypes were small in the darkness, they were very large under illumination. These data suggested that the resistance mechanism may be light-dependent.

The effect of diquat and paraquat on chlorophyll *a* fluorescence was also investigated. The ability of the bipyridyls to accept electrons at PSI keeps the

electron transfer chain oxidized and thus suppresses fluorescence. Thus, fluorescence is a direct measure of the presence of bipyridyl cations at the site of action. With both paraquat and diquat, chlorophyll fluorescence was reduced at the same rate in both biotypes. This indicated that both paraquat and diquat penetrated to the site of action at the same rate. However, the magnitude of the fluorescence quenching was smaller in the R biotype. Concentration-dependent chlorophyll fluorescence was also examined. Greater concentrations of diquat and paraquat were required by the R biotype in order to achieve similar levels of fluorescence quenching. Because control levels of fluorescence tended to be higher in the R biotype, the data were interpreted to indicate that paraquat resistance was probably due to reduced electron transport or reduced affinity for bipyridinium ions at the site of action.

Taken together, ethane production, ion leakage and fluorescence showed that paraquat penetrated to the site of action, that the active site was capable of reducing both diquat and paraquat and that sufficient oxidative stress was generated in the R biotype to induce phytotoxicity, although at a lower level than the S biotype. However, they do not unequivocally eliminate the possibility that less paraquat may penetrate to the target site or that a target site mutation may have occurred. The ethane assays did eliminate elevated levels of protective enzymes from being the primary mechanism because KCN was used to inhibit SOD and ascorbate peroxidase and a differential response was still obtained.

In order to test whether the primary mechanism was chloroplastic or cytoplasmic, assays for differential electron transport and O_2^- production were

conducted with thylakoid membranes isolated from intact chloroplasts. Photosynthetic electron transport by nontreated thylakoids occurred at more rapid rates in the S biotype than the R biotype. Both biotypes produced significant amounts of O_2^- when thylakoids were illuminated without paraquat. However, the rate of O_2^- production was greater in the S biotype. The addition of paraquat had a greater effect on the rate of radical production of the S biotype. Paraquat-treated thylakoids of the R biotype and nontreated thylakoids of the S biotype had similar rates of O_2^- production. Thus low rates of electron transport and correspondingly low levels of oxidative stress in nontreated thylakoids of the R biotype provided evidence that the resistance mechanism involves chloroplast photochemistry. Ferricyanide, a Hill reagent, is thought to accept electrons at a site located before paraquat's target site. Therefore, paraquat resistance in American black nightshade is probably due to reduced electron transport rather than a target site mutation. The fluorescence data can now be reassessed to indicate that the mutation causing reduced electron transport is located on the reducing site of PSII.

Based on the results of these physiological and biochemical studies that were conducted to investigate the mechanism of paraquat resistance in American black nightshade, the null hypothesis was rejected. Paraquat resistance cannot be explained by a previously reported mechanism. Therefore, the alternative hypothesis, paraquat resistance is due to a unique and previously unreported mechanism, was accepted.

It may be argued that since the ascorbate-glutathione cycle requires NADPH as a source of reducing power, paraquat must be removed from the site of action and its

effect neutralized in some manner in order for plants of the R biotype to continue normal growth. This may not be necessary in American black nightshade because the levels of O_2^- in the paraquat-treated R biotype did not exceed the level of O_2^- in the untreated S biotype. Unpublished work by T.A. Bewick and K.L. Smith appeared to indicate that constitutive levels of antioxidants were the same in both biotypes and that after an initial decrease during the first hour of paraquat treatment, antioxidant levels in the R biotype were stimulated to levels in excess of the constitutive levels. It is possible that sublethal levels of paraquat may actually improve the efficiency of electron transport so that the $NADP^+$ reduction is not significantly affected. Alternatively, it is possible that like many toxic substances that may occur in plants cells, paraquat is gradually pumped into and stored in the vacuole.

The screening of progeny to select individuals homozygous for paraquat resistance and sensitivity should be completed, and the mode of inheritance of the resistance trait investigated. Future research should be performed using progeny of the homozygous selections. Such research should involve the corroboration of reduced electron transport at whole plant level. Electron paramagnetic spectroscopy can be used to determine, unequivocally, whether the site of action has changed. The use of a series of electron donors and acceptors can be employed with thylakoids in order to determine the actual site of the mutation. The characterization of a mutation that results in reduced electron transport could potentially contribute significantly to photosynthesis research.

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BIOGRAPHICAL SKETCH

Carlene is a citizen of Trinidad and Tobago. She attended The University of the West Indies (UWI) where she was the recipient of an L.J. Williams bursary and an award from the West Indies Group of University Teachers. She graduated in 1986 with a Bachelor of Science degree in agriculture with honors. She then worked for one year as a research assistant with UWI on a cooperative project with CAB International Institute of Biological Control and Caroni (1975) Limited. Her work involved the biological control of the sugarcane froghopper and she is co-author of a paper on the subject.

In 1987, Carlene began work with Dr. Vincent Souza Machado at the University of Guelph as a Canadian Commonwealth Scholar. Her project examined the effect of a growth regulator on the size and yield of seed potatoes. In 1988, she was awarded a Hoskins fellowship for academic excellence. Carlene was also a member of the weed team that competed at the North-Eastern Weed Competition in 1988. She completed requirements for a Master of Science degree in horticultural science in 1989.

Carlene came to the University of Florida in Spring of 1990 and has worked in close association with her advisor Dr. T.A. Bewick and Dr. D.G. Shilling. She has had the opportunity to teach Horticultural Plant Identification and Weed Science

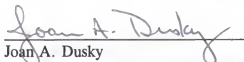
laboratories. In 1992, Carlene was inducted into Gamma Sigma Delta, the honor society of agriculture and in 1994 was the recipient of the Florida Weed Science Society's Outstanding Graduate Student Award. She is a member of the Weed Science Society of America, the Southern Weed Science Society and the Florida Weed Science Society.

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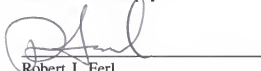
Thomas A. Bewick, Chair
Associate Professor of Horticultural
Science

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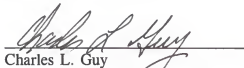
Joan A. Dusky
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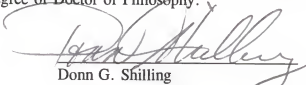
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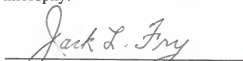
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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